UNIFORMED SERVICES UNIVERSITY OF THE HEALTH SCIENCES
F. EDWARD HEBERT SCHOOL OF MEDICINE
4301 JONES BRIDGE ROAD
BETHESDA, MARYLAND 20814-4799

GRADUATE EDUCATION
(301) 295-3913
FAX: (301) 295-6772

APPROVAL SHEET

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Name of Candidate: Sidney Palmer
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Dissertation and Abstract Approved:

Linda Porter, Ph.D. — 8/3/99
Department of Anatomy & Cell Biology
Committee Chairperson

Sharon Juliano, Ph.D. — 8/3/99
Department of Anatomy & Cell Biology
Committee Member

Denes von Agostin, M.D., Ph.D. — 08/03/99
Department of Anatomy & Cell Biology
Committee Member

MAJ Ajay Verma, USA — 08/05/99
Department of Neurology
Committee Member

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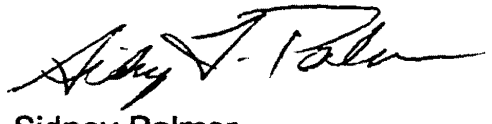

Sidney Palmer
Department of Anatomy and Cell Biology
Uniformed Services University of the Health Sciences

Abstract

Title of Dissertation: The Role of Layer 4 in Thalamocortical Development

Sidney Lorin Palmer, Doctor of Philosophy, 1999

Thesis Directed by: Sharon L. Juliano, Ph.D.

Professor of Anatomy and Cell Biology and Neuroscience

Thalamic innervation of cortex is a complex process in which specific regions of the thalamus must grow into precise cortical regions. Once within a specific cortical area, thalamic afferents terminate largely upon a target population of cortical cells. Within the somatosensory cortex, thalamic afferents terminate largely upon layer 4 cells. To further understand the importance of layer 4 in shaping the development of thalamic afferents and the specificity of their connectivity within somatosensory cortex, we disrupted the formation of layer 4 of ferret somatosensory cortex using the anti-mitotic drug, methylazoxy methanol acetate (MAM) on embryonic day 33 (E33). As a control, MAM was administered to a second group of animals on E38 to interfere with layer 2 formation. Following MAM treatment, two sets of experiments were performed. In the first set of experiments, small crystals of the lipiphilic tracer, DiI were placed into the ventral basal (VB) region of thalamus of fixed brains obtained at P1, P7 and P14. The distribution of thalamic fibers within the somatosensory

cortex was analyzed at each age. In the second set of experiments, organotypic cocultures were created composed of P0 normal thalamus paired with normal, E33 or E38 MAM-treated cortex obtained from kits at P0 or P7. Injections of anatomical tracers were made into the thalamic pieces of the cocultures and the resulting thalamic fibers and their terminations were analyzed with regard to their position of termination within the host cortex. Findings from the *in vivo* experiments demonstrate a pattern of development in normal cortex where thalamic afferents grow into developing cortical plate and begin the process of stopping and terminating by P1. By P7, many of the thalamocortical fibers have stopped within layer 4, and by P14, a distinct band of terminal fibers is apparent in layer 4. This pattern of development is disrupted in E33 MAM treated cortex. Thalamic fibers at all ages examined demonstrate different distributions. Significant differences are noted at P7 and P14 in which significantly higher numbers of fibers grow beyond the cortical plate and layer 4 region and into more superficial regions. Thalamic afferents in E38 MAM-treated cortex demonstrate a developmental pattern of ingrowth and termination similar to normal. These results were similar to those observed in the organotypic coculture environment. Analysis at 3, 5, and 7-10 days in culture (dic) reveal that thalamic ingrowth into normal cortex show specificity of termination within cortical plate. Conversely, thalamic afferents growing into E33 MAM-treated cortex at each of these times in culture demonstrate significant differences in terminations, with many fibers growing beyond the cortical plate and into deeper cortical

layers. Thalamic fiber terminations in E38 MAM-treated host cortex reveal a pattern of distribution similar to normal. Taken together, the results of these experiments clarify the apparent importance of layer 4 in providing organizational cues to thalamic afferents, especially, the role layer 4 plays in instructing thalamic afferents to stop and form appropriate terminations.

THE ROLE OF LAYER 4 IN THALAMOCORTICAL DEVELOPMENT

by

Sidney Lorin Palmer

Dissertation submitted to the Faculty of the
Department Of Anatomy and Cell Biology of the
Uniformed Services University of the Health Sciences
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Doctor of Philosophy 1999

Dedication

To our parents, John and Lorine Palmer and Leslie and Bonnie Smart, to whose strong roots we have returned frequently for strength and sustenance. They have been and continue to be an anchor to my family and have taught me by word and deed that despite the great things we may accomplish in life, our most important work continues to be done at home.

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remarkable teacher. I am grateful to Dr. Rosemary Borke, who is an excellent teacher and example, and to Dr. Juanita Anders who has been so supportive and encouraging over the years.

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Table of Contents

Approval Sheet i

Copyright Statement ii

Abstract iii

Title Page vi

Dedication vii

Acknowledgements viii

Table of Contents x

Introduction 1

Cortical Development 1

Ferret Model 3

Development of Thalamocortical Projections 4

Paper 1: Disruption of layer 4 during development leads to altered thalamocortical projections in ferret somatosensory cortex. 8

Abstract 9

Introduction 11

Methods and Materials 14

Experimental Design. 14

MAM Treatment and Disruption of Layer Formation. 14

DiI Injections 15

Analysis 15

Results 18
Thalamocortical projections in E33 Normal Cortex 18
Thalamocortical Projections in normal and MAM-treated brains at P1 19
Thalamic projections in normal and MAM-treated brains at P7 20
Thalamic projections in normal and MAM-treated brains at P14 21
Measurements of ventrobasal thalamus 22
Discussion 23
Summary 23
The use of the MAM 23
What is the nature of neocortical influence on thalamic termination? 27
What is the nature of layer 4 influence? 28
How does diminution of layer 4 affect function? 30
Bibliography 31
Figure Legends 39
Paper 2: Laminar specific alterations of thalamocortical projections in organotypic cultures following layer 4 disruption in ferret somatosensory cortex 53
Abstract 54
Introduction 56
Methods 59
Organotypic Cocultures 59
MAM Treatment and Disruption of Layer Formation 60
Anatomical Tracer Injections 61

Analysis 62
Results 64
Characteristics of normal cortical organotypic cultures 64
Distribution of thalamic afferents 67
Three Days in Culture 67
P0 cortex paired with P0 thalamus 67
P7 cortex paired with P0 thalamus 68
Five Days in Culture 69
P0 cortex paired with normal P0 thalamus 69
P7 cortex paired with normal P0 thalamus 70
Seven -Ten Days in Culture 71
P0 cortex paired with P0 normal thalamus 71
P7 cortex paired with P0 thalamus 72
Discussion 73
Summary 73
Effect and specificity of MAM treatment 74
The organotypic culture environment 74
The influence of cortical maturation 76
What is the role of layer 4? 77
Bibliography 80
Figure Legends 86
Summary 107

Factors Involved in Thalamic Growth and Target Selection 107

Bibliography 112

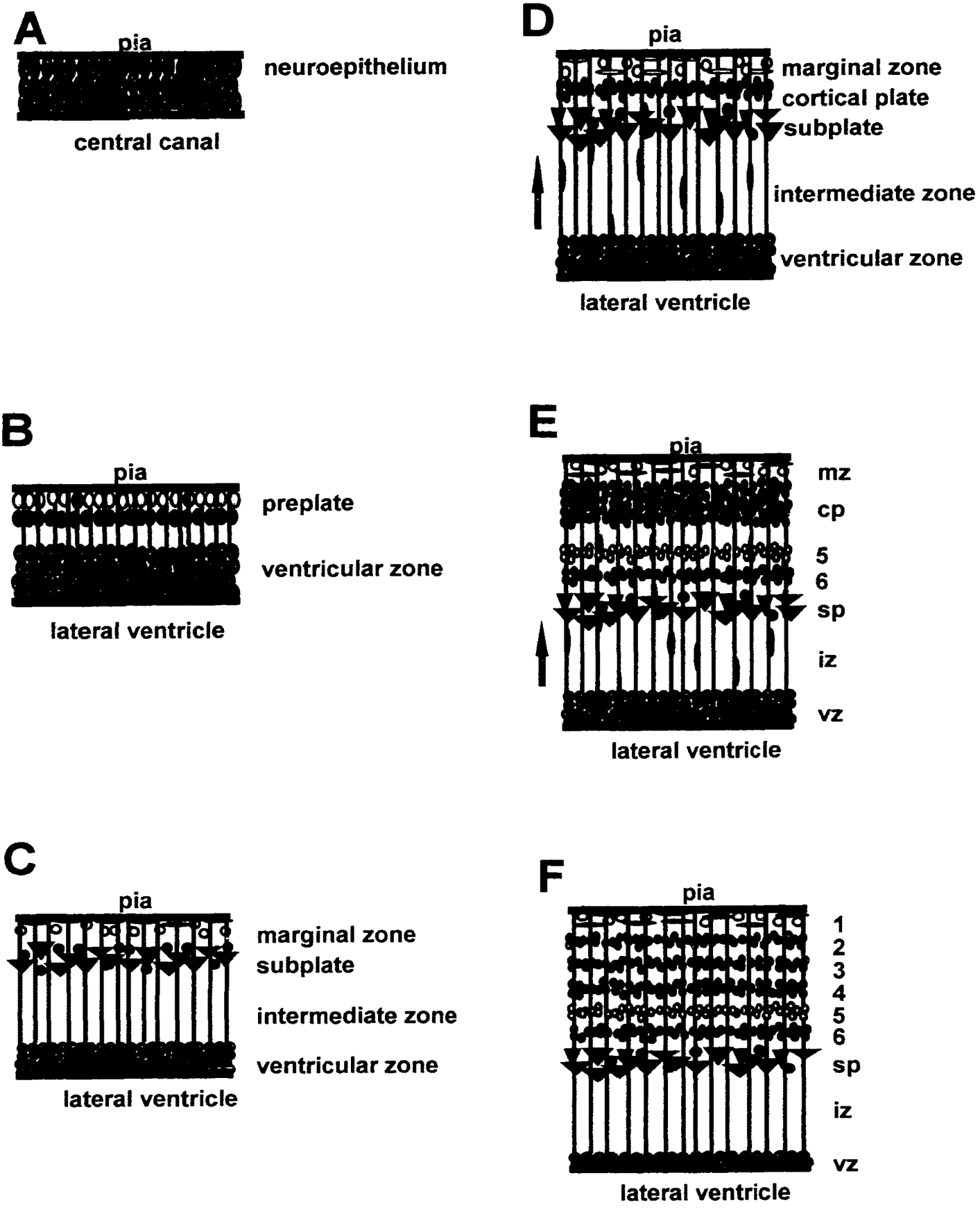
Introduction

Cortical Development

The thalamus, the major relay nucleus of the nervous system, is responsible for shuttling information from most of the sensory organs directly into the cerebral cortex. The neocortex is composed of numerous cytoarchitectonic areas, differing in connectivity and functional properties. The precise input of thalamic afferents into these diverse areas to a large extent, shapes cortical function and structure properties within that region of cortex (O'Leary et al., 1994). Specific nuclei within the thalamus project to very distinct regions of the cortex and terminate largely on a precise target cell population. Within the somatosensory cortex, layer 4 receives the majority of the thalamic input (Jones and Burton, 1976). The mechanisms that direct the growth of thalamic fibers into neocortex and cause their proper termination have been an area of intense research. So closely tied are the functions of the cortex to the afferent input from the thalamus that attempts to clarify their development are best considered together.

The developing neocortex begins as a rudimentary structure composed of pseudostratified proliferative neuroepithelium (ventricular zone) that lines the forebrain vesicle (Figure 1). The cells generated earliest in the mitotically active ventricular zone form the preplate, composed of newly born cells that accumulate against the edge of the cerebral wall. Cells born later form the early

Figure 1 .Illustration of the events involved in corticogenesis. (A) Early neuroepithelium surrounding the central canal of the nervous system. (B) Formation of earliest generated cells, forming the outer preplate regions. (C) Generation and migration of the transient cell population, the subplate, which splits the preplate into a marginal zone (layer1) and an intermediate zone. (D-F) The formation and migration of cells populating the cortical layers, proceeding in an in-side-out fashion. mz=marginal zone; cp=cortical plate; sp=subplate; iz=intermediate zone; vz=ventricular zone.


A
pia
neuroepithelium
central canal
D
pia
marginal zone
cortical plate
subplate
intermediate zone
ventricular zone
lateral ventricle
B
pia
preplate
ventricular zone
lateral ventricle
E
pia
mz
cp
5
6
sp
iz
vz
lateral ventricle
C
pia
marginal zone
subplate
intermediate zone
ventricular zone
lateral ventricle
F
pia
1
2
3
4
5
6
sp
iz
vz
lateral ventricle

cortical plate as they migrate away from the ventricular zone and split the preplate into an outer marginal zone (cortical layer 1) and an inner subplate region. Successive waves of subsequently generated neurons migrate away from the proliferative ventricular zone, through the subplate neurons and the previously established cortical plate neurons to settle below the marginal zone. The cortical plate neurons accumulate in an inside out fashion; each wave of arriving cells migrates through the existing cells of the cortical plate before taking up a superior position (Lund and Mustari, 1977; Rakic, 1978). The early arriving lower cortical plate neurons are the first to mature and differentiate to form layers 6 and 5. Younger neurons arrive in the cortical plate and differentiate into layers 4, 3 and 2 (Bayer and Altman, 1991).

Ferret Model

Ferrets as an animal model are important for examining the development of the nervous system. Ferrets are born with a relatively immature nervous system that undergoes a protracted period of development (Jackson et al., 1989). Much of the development and maturation of the cortex occurs within the late embryonic and early postnatal period. Cells that populate the somatosensory cortex are generated over a period of several days with the peak of cell division for any given layer occurring within approximately a 24 hour period (Noctor et al., 1997).

Experiments performed in this lab confirm that ferret somatosensory cortex develops in an inside out fashion typical of mammalian neocortex, but

follows a somewhat different developmental time line than other regions of the developing ferret cortex (Jackson et al., 1989; Noctor et al., 1997). Birthdating studies using the thymidine analog, 5-bromo 2-deoxyuridine (BRDU), indicate that unlike visual cortex in which many neurons continue to be generated after birth, most components of the somatosensory cortex are already born by the day of birth (Jackson et al., 1989; Noctor et al., 1997). Many of the already generated cells belonging to the somatosensory cortex continue to migrate into the cortex after birth and a cohort of cells that belong to layer 2 are still being generated.

Development of Thalamocortical Projections

The arrival of thalamic afferents into neocortex occurs before the layers are fully established. Many of these afferents "wait" in the subplate region before entering the cortex. Thalamic axons begin to invade the cortical plate, following the "waiting period", which corresponds with layer 4 cell migration into their final position. The thalamic axons grow through the previously established layers 5 and 6 and arborize within layer 4 during the next few weeks of development (Rakic, 1977; Allendoerfer and Shatz, 1994). In the visual cortex of several mammalian species, projections from each eye through the LGN share a high degree of overlap into neighboring ocular dominance columns. The overlapping patches of terminal arbors from each eye undergo refinement during normal development, leading to precisely segregated patches of thalamic terminals from each eye (Antonini and Stryker, 1993). The patchy terminations in somatosensory regions, however, appear to require comparatively little "pruning"

and are relatively focused on arrival (Catalano et al., 1991; Agmon et al., 1993; O'Leary et al., 1994; Schlaggar and O'Leary, 1994; Juliano et al., 1996). In the visual cortex, however, afferents from the lateral geniculate require a combination of elimination of nonspecific collaterals and directed outgrowth into appropriate sites to produce normal, adult-like discrete patches (Antonini and Stryker, 1993).

One way to observe the interactions between the developing neocortex and the arriving thalamic afferents is to selectively remove or lesion specific layers of the cortex. Ghosh and Shatz (1994) used kainic acid to lesion developing cortex and observed that thalamic innervation was disrupted and thalamic axons failed to find their appropriate target layer. Although this method has produced very interesting results, at the time of injection, much of cortical development has already occurred. In addition, thalamocortical connections are establishing and the cytotoxic effect of kainic acid may disrupt other neural components besides the targeted layer. Furthermore, the cytotoxic nature of kainic acid affects more mature neurons (Ghosh and Shatz, 1993). Because of this, it is difficult to target specific layers other than subplate. By the time neurons of the cortical plate, such as layer 4, are sufficiently mature to be affected by kainic acid, earlier maturing layers 5 and 6 would also be affected. Ionizing radiation has also been used to lesion specific cortical layers. Algan and Rakic (1997) demonstrated that differing doses of irradiation delivered to macaque monkeys at specific embryonic dates effectively reduced or removed specific

cortical layer populations.

The timing and duration of corticogenesis in ferrets make it particularly well suited for use in studying cortical deletions using an antimitotic that prevents cell division for a restricted period, methylazoxymethanol acetate (MAM). Derived from the cycad plant endogenous to the Mariana Islands, MAM has been shown to be an effective agent for selectively diminishing specific populations of cells (Yurkewicz et al., 1984; Woo and Finlay, 1996; Noctor, 1998; Noctor et al., 1999). By methylating guanine residues in DNA, MAM blocks the action of the DNA polymerase molecule and inhibits both protein synthesis and mitotsis (Matsumoto and Higa, 1966; Zedeck et al., 1970; Cattabeni and Di Luca, 1997). Mitotically active cells are eliminated for approximately 12 hours (Evans and Jenkins, 1976; Noctor, 1998). Careful timing of the administration of this drug during ferret corticogenesis can selectively lesion developing cortical layers. MAM has also been used as a successful lesioning agent in other species and has interfered with the development of cortical layers in rats and hamsters (Johnston et al., 1979; Jones et al., 1982; Yurkewicz et al., 1984; Virgili et al., 1988; Woo and Finlay, 1996). Most of the previous experiments using rodents did not attempt to specifically lesion a single layer, and the resulting cortical disruption involved multiple layers. In rats, cortical development proceeds relatively rapidly so several cortical layers may be generated on a given day and a single MAM treatment *in utero may* affect the development of more than one cortical layer. The resulting lesion involves several layers. In ferrets, however,

the duration of the peak of neurogenesis for a single cortical cell layer coincides with the length of activity of MAM, and layer-specific lesions are possible.

In the following papers, the developmental interactions between the thalamus and the cortex are explored. Specifically, I assess the role of layer 4 in cortical development. We ask the question, "Is layer 4 required for normal thalamocortical innervation and maturation?" In the first paper, we observe the development of thalamic afferents in normal cortex and the cortices of ferrets in which corticogenesis was interrupted on embryonic days (E)33 or 38. In the second paper, we explore these same interactions in the organotypic coculture environment wherein we pair E33 or E38 disrupted cortex with normal thalamus and allow them to grow together. We know from earlier experiments that interfering with cortical development on E33 disrupts layer 4, whereas interfering on E38 disrupts layer 2 (Noctor et al., 1997). Thus these papers are concerned with the development of thalamocortical interactions in the relative absence of layer 4.

DISRUPTION OF LAYER 4 DURING DEVELOPMENT LEADS TO ALTERED THALAMOCORTICAL PROJECTIONS IN FERRET SOMATOSENSORY CORTEX.

Sidney L. Palmer, Stephen C. Noctor, Sharon L. Juliano

Department of Anatomy & Cell Biology, USUHS, Bethesda, MD 20814

Abstract

Identifying the factors that influence the precision of the projections from the dorsal thalamus to the neocortex are important toward understanding the overall organization and function of the cerebral cortex. Since layer 4 receives the bulk of thalamic afferent projections, comprehending the contribution and influence of this layer on thalamic terminations is significant. To more clearly identify the role of layer 4 in forming the thalamic afferent projection pattern in the somatosensory cortex, we disrupted the birth of this layer during corticogenesis and studied the resulting pattern of thalamic terminations. To do this, methylazoxy methanol (MAM) was injected into pregnant ferrets on gestation day 33 (E33), the date that most of the neurons destined to populate layer 4 of somatosensory cortex are generated. For comparison purposes, MAM injections were also made on E38, the day that neurons destined to populate layers 2-3 are generated. After birth of the ferret kits, the somatosensory cortex was analyzed at postnatal day 1 (P1), P7, and P14, at which time injections of DiI were made into the ventrobasal thalamus. We found little differences between groups in the thalamic afferent pattern at P1; at this date, the thalamic afferents terminated in the lower part of the immature cortex, mostly corresponding to the poorly formed layers 5 and 6. By P7, significant differences in the terminal thalamic pattern emerge, when the E33 MAM-treated group is compared to either the normal or the E38 MAM-treated animals. More

terminations can be seen in layer 1 and the undifferentiated cortical plate in the E33 MAM-treated animals. By P14 there are substantial differences between the distribution of thalamic afferents in the E33 MAM-treated animals compared to normal or E38 MAM-treated ones. At this age, the thalamic terminations are almost equally distributed throughout all the remaining cortical layers in the E33 MAM-treated group, as contrasted to the normal and E38 MAM-treated animals, in which the ventrobasal thalamus projected primarily to central layers. These findings emphasize the importance of layer 4 in determining the normal pattern of thalamic termination, and suggest its absence may impact significantly on the overall functional responses of the neocortex.

Introduction

The precise patterning of thalamic afferent growth into specific cortical layers is largely responsible for the explicit function and structure of the cerebral cortex. Each cytoarchitectonic field is formed partly by its intrinsic architecture and connections, and in part by the specific interactions between the thalamus and cortex. The mechanisms that direct the growth of thalamic axons into the neocortex and guide growing fibers into their site of proper termination have been a subject of intense interest for a number of years (e.g., Hubener et al., 1995; Mann et al., 1998; Molnar et al., 1998). In most primary sensory neocortical areas, a principal terminal site of the specific thalamic nuclei is layer 4. A number of recent studies attempt to define factors that are "special" about layer 4 and why most thalamic fibers choose to stop there. Layer 4 may serve a chemotrophic function that attracts growing thalamic fibers or may also provide a stop signal to incoming thalamic afferents instructing them to terminate in that site (Molnar et al., 1991; Gotz et al., 1992; Bicknese et al., 1994; Hubner et al., 1995; Miller et al., 1995; Yamamoto et al., 1997; Molnar et al., 1999; Molnar and Blakemore, 1999).

To further define the influence of layer 4 on the ultimate position and function of thalamic terminations, we conducted a study that disrupted the development of this layer, using injections of methylazoxy methanol (MAM) during gestation of the ferret. By using appropriately timed injections, MAM

treatment leaves the maturing cortex diminished of neurons that usually populate layer 4. We previously determined that MAM injections on embryonic day 33 (E33), in ferrets, dramatically reduces the size and number of cells in layer 4 of primary somatosensory cortex (area 3b), with minimal disruption to the other layers (Noctor, 1998; Noctor et al., 1999). We also reported that injections of MAM on other gestational days result in highly specific alterations of laminar development, depending on whether the drug is delivered early or later during cortical development (Noctor et al., 1999). Using this technique of layer disruption allowed us to study thalamocortical connections in the relative absence of layer 4. MAM prevents DNA synthesis in dividing cells by methylation of the 7' position of guanine (Matsumoto and Higa, 1966; Zedeck et al., 1970; Cattabeni and DiLuca, 1997). The DNA methylation results in reduced nucleic acid synthesis, due to inhibition of DNA polymerase, which ultimately results in decreased protein synthesis. This produces a decrease in cell division, which recovers in 8-12 hours (Noctor et al., 1999; Evans and Jenkins, 1976).

Other studies have used MAM to interfere with the development of specific neocortical layers in different species. The earlier reports indicate that interrupting the generation of cortical cells in rats and hamsters, including layer 4, results in projections from the thalamus that terminate in a bilaminar pattern (Yurkewicz et al., 1984; Woo and Finlay, 1996). In other words, the overall pattern is relatively typical, with the remaining layers receiving the input intended for layer 4 in a quasi-normal laminar pattern. In our experiment, the thalamic

projections did not resemble the normal distribution but spread almost equally throughout the remaining cortical layers. In the study presented here, pregnant ferrets were injected with MAM, and after the ferret kits were born, thalamocortical projections were evaluated at different ages along with age matched normal animals. We also studied MAM-injected animals during a gestational age that was timed to interrupt the development of layer 3-2. We report that interruption of layer 4 development using MAM in ferrets results in altered projections from the ventrobasal thalamus (VB). Rather than terminating in a focussed pattern in the vicinity of layer 4, VB projects diffusely to all cortical layers with many fibers terminating in upper layers.

Methods and Materials

Experimental Design.

Normal, embryonic day 33 (E33) MAM treated, and E38 MAM treated ferrets were used at 3 different ages: P1, P7, & P14. During pregnancy, ferrets were injected with MAM on either E33 or E38. After the kits were born, the brains were removed, blocked, and DiI injections placed in the ventrobasal thalamus. After an appropriate period to allow for diffusion of the tracer, the brains were cut and analyzed to assess the distribution of label found in the cerebral cortex. Also included in the analysis were fetuses from two pregnant ferrets, which were removed by caesarian section at E33; these fetuses were otherwise normal. This study focussed on projections that terminated in primary somatosensory cortex.

MAM Treatment and Disruption of Layer Formation.

Pregnant ferrets were anesthetized with 5% halothane and 0.05% N_2O. An injection of methylazoxy methanol acetate (Sigma; 12mg/kg) was administered IP on either E33 or E38. The MAM injections disrupted cells undergoing final mitosis that were intended for either layer 4 (E33) or layers 2-3 (E38). We previously determined the precise days of gestation for cells populating specific layers of ferret somatosensory cortex and MAM injections on these dates prevent a large percentage of either layer 4, or layers 2-3, from being formed (Noctor, 1998; Noctor et al., 1999).

DiI Injections.

Each ferret at the appropriate age, P1, P7, or P14 received an IP injection of Pentobarbital Na (50 mg/kg). When insensitive to pain, the animal was transcardially perfused with saline followed by 4% paraformaldehyde in 0.1M phosphate buffer. The brains were removed, blocked, and placed in the same fixative at 4°C until DiI was injected. At the time of the DiI injection, the blocks were trimmed coronally from the posterior aspect to the level of the posterior thalamus. A crystal of DiI was inserted into the ventrobasal nucleus of the thalamus under microscopic guidance using a pipette. Following an incubation period of 6-8 weeks, each cortical hemisphere was embedded in 3% agar and cut at 100 μm thickness in the coronal plane on a Vibratome. Each slice was mounted on a gelatin subbed slide and counterstained with a 0.2% bisbenzimide solution in phosphate buffer for 2 minutes, then coverslipped with fluorescent mounting media.

For two pregnant ferrets, fetuses were removed using sterile conditions by caesarian section on E33 while the animals were anesthetized with fluothane (2-5%). Brains from the fetuses were removed and placed in fixative (4% buffered paraformaldehyde); they were stored at 4°C until they received DiI injections as described above.

Analysis.

The sections were analyzed and the labeled pathways reconstructed

using fluorescent microscopy and the image data collection software Image Pro (Digital Solutions) and Neurolucida (Microbrightfield). All slices were examined on a fluorescent microscope outfitted with a CCD camera. Images were collected from each focal plane within an area of interest. These images were summed together to produce a flattened image representing the entire 100 μm thickness of a slice. We then imaged the same area of interest with the fluorescent bisbenzimide counterstain. From this image, we determined the cortical layers based on cell morphology and nuclear densities. The boundaries of the cortical layers were superimposed on the flattened image of the thalamic afferents. With the boundaries of the cortical layers in place, we counted the number of afferent fibers found within each identified boundary. Since different brains were more or less densely labeled by the DiI, results per layer were expressed as a percent of total fibers within layers. These percentages were averaged between slices of the same brain to give a representative average for each layer for a specific brain. Each labeled fiber was traced to its origin within a given section. This was to insure that processes issuing from retrogradely labeled cells in the cortex were not mistaken for thalamic afferent fibers. A Mann-Whitney-U test was performed on the averages, comparing normal to E33 or E38 MAM-treated at each age. The E38 MAM-treated brains were excluded from statistical analysis at two ages since there were fewer of these brains than either E33 MAM-treated or normal. The main intent of including the E38

treatment was as a control to validate the specificity of the E33 MAM treatment.

In a representative set of animals, we assessed the volume of VB thalamus in normal and MAM-treated animals to verify that MAM injections on E33 did not alter the size and viability of the VB. To do this, the rostral border of the nucleus was delineated on horizontal sections and used landmarks observed in this plane as a guide for definitive identification in coronal sections. Although a ferret atlas does not exist, many of the features of the ventrobasal nucleus are similar to those in cat, and the atlas of Berman and Jones (1982) was used as a guide. We included in our assessment nuclei identified in their nomenclature as ventrobasal, arcuate (VBA), ventrobasal, external (VBX), and basal ventromedial (VMB). We refer to them collectively as the ventrobasal complex (VB). After identifying the rostral border on horizontal sections, we could easily identify VB in coronal sections stained with bisbenzimide. We delineated the borders of VB in the 5 most rostral sections (i.e., over 500 μm) of normal and E33 MAM-treated thalami. The boundary of this nucleus was outlined, and the volume determined in normal and MAM-treated brains using ImagePro.

Results

This study evaluated DiI label found in the somatosensory cortex resulting from injections in the ventrobasal thalamus of normal and MAM-treated ferrets at different ages. A total of 17 pregnant ferrets were used, 7 were normal, 7 received MAM injections on E33, 3 received MAM injections on E38. Of the offspring, 33 kits were used at different ages (see Table 1). The DiI injections were fairly large and some label diffused from the injection site into other regions of thalamus (Figure 1). Our analysis was restricted to the somatosensory cortex. Although it was not possible to precisely identify somatosensory cortical areas by cytoarchitecture at the ages studied, morphologic landmarks were reliable indicators of the region to be studied (Juliano et al., 1996). We included for quantitative study a restricted block of cortex approximately 600 μm wide in a site immediately posterior and lateral to the post cruciate dimple (Figure 2). This landmark was visible at all ages studied; the identified region corresponds to the hand region of area 3b in the adult (Juliano et al., 1996; McLaughlin et al., 1999).

Thalamocortical projections in E33 Normal Cortex

Injections were made at this age to demonstrate the maturity of the brain at the time of the E33 MAM injection. All further definition of cortical architecture occurs after MAM treatment. Cytoarchitecture of the cortex can be seen using the nuclear label bisbenzimide (Figure 3). At this age, a cell-sparse layer 1 is evident along with a densely populated, but immature cortical plate. Cortical

layers cannot be distinguished. The subplate and ventricular zones are also clearly visible at E33. When DiI was injected into the lateral portion of the thalamus (VB was not fully differentiated at this age), the resulting label showed thalamic afferents growing into the cortex, forming a densely labeled thalamocortical bundle superior to the ventricular zone. Many of the thalamocortical fibers left this collection and extended toward, but rarely reached the subplate. None of the labeled fibers left the subplate region to enter the overlying immature cortical plate.

Thalamocortical Projections in normal and MAM-treated brains at P1

At this age in both the normal and MAM-treated cortex, the developing layers were poorly distinguished. The developing cortical plate consisted of the poorly defined layers 5 and 6 and the cell-dense, undifferentiated layers 2-4 (Figure 4). Layer 1 is also present, as is a distinct subplate (Juliano et al., 1996; Noctor, 1998). There were few differences in the appearance of the cytoarchitecture between treatment groups, although the cortex was slightly thinner in the E33-MAM treated cortex, but the thickness of the E38 MAM-treated cortex was similar to the normal (Figure 5) (Juliano et al., 1996; Noctor, 1998).

In the normal ferret cortex at P1 labeled afferent fibers occur in lower layers 5 and 6 and subplate region. In E33 and E38 MAM-treated cortex, the majority of DiI labeled thalamic fibers are also found within the lower layers of cortical plate. Occasional labeled fibers appear within the undifferentiated cortical plate in all treatment groups, although they usually do not end there, but

ultimately terminate in layer 1. Many of the labeled afferent thalamic fibers travel tangentially in the white matter and immature lower cortical layers, as has been reported by a number of other groups, but turn to enter the cortical plate with a vertical approach (Catalano et al., 1991; Agmon et al., 1993; Juliano et al., 1996). In the quantitative analysis, the number of fibers counted in each layer was expressed as a percent of the total number of labeled fibers. At this age, P1, the following layers were identified for analysis: layer 1, the dense, undifferentiated cortical plate (CP), layer 5 and layer 6 (Figure 6). The quantitative analysis revealed no significant differences between groups in the distribution of total fibers within the analyzed layers (Figure 7; Mann-Whitney U Test).

Thalamic projections in normal and MAM-treated brains at P7

At P7, the cytoarchitecture of somatosensory cortex is similar to that seen at P1 (Juliano et al., 1996). The cortical mantel is thicker and layers 5 and 6 are more easily distinguished. The laminar features are otherwise comparable to those observed on P1; there are no significant differences in thickness (Figures 4 & 5). By P7 in thalamocortical development, significant differences begin to emerge between normal and E33 and E38 MAM-treated brains (Figure 6). Qualitatively, more apparent branching is observed in the developing cortical plate of normal brains. Many thalamic afferents enter the cortical plate and turn to begin forming arbors. Less branching is apparent in the E33 MAM-treated brains.

Quantitative analysis of percentages of the total numbers of labeled fibers finds that in normal animals and E38 MAM-treated brains, the greatest percentage of fibers are found in the deeper layers (Figure 8). These afferent fibers are most likely growing to terminate in layer 4. In the E33 MAM-treated brains relatively fewer labeled afferent fibers are found in layer 5, and significantly more fibers are distributed in the cortical plate ($p=0.048$) and layer 1 ($p=0.012$) compared to those distributions in normal animals.

Thalamic projections in normal and MAM-treated brains at P14

The most obvious differences between normal and E33 MAM-treated brains occur at P14. At this age, the normal laminar architecture is more distinct and each cortical layer can be identified although they have not attained complete maturity (Figure 4) (Juliano et al., 1996). In the MAM-treated animals, the specific layer targeted is diminished in dimension (Noctor et al., 1999). For this analysis, we identified the following laminar distinctions: layer 1, upper cortical plate (presumptive layer 2-3), lower cortical plate (presumptive layer 4), and layers 5 and 6. The thickness of the somatosensory cortex is significantly thinner in the E33 MAM-treated animals compared to normal (Figure 5; two-tailed t-test, $p=0.004$). Organization of thalamic afferents is strikingly different between E33 MAM-treated and control cortices (Figure 6). In the normal somatosensory cortex, the greatest percentage of DiI labeled thalamic afferent fibers distribute in the lower layers (5&6) and in the lower cortical plate. Very few fibers extend beyond this point. Many labeled thalamic fibers projecting into the

cortex in the E33 MAM-treated animals fail to terminate within the lower layers or lower cortical plate and continue into upper layers, including layer 1. This distribution pattern in the E33 MAM treated brains present relatively equal percentages of fibers terminating across cortical layers, compared to the bulk of fibers that are found in the lower cortical plate and layer 5 in the normal and E38 MAM-treated. These data achieve statistical significance (Figure 9).

Measurements of ventrobasal thalamus

To determine that MAM treatment timed to interfere with layer 4 development did not dramatically alter the nature of the thalamus projecting to the somatosensory region, we measured the volume of VB in normal and MAM-treated animals. This analysis determined that there were no significant differences in the size of VB at any of the ages examined: P1, P7, and P14 (Table 2).

Discussion

Summary

We investigated the impact of reducing layer 4 on the subsequent growth of thalamic axons into the somatosensory cortex of ferrets. Interference with the development of layer 4 using appropriately timed injections of MAM results in the altered distribution of thalamocortical projections. Projections from VB are almost uniformly distributed among all neocortical layers when layer 4 is diminished, rather than being focussed in the central region . MAM injections timed to interfere with the development of layer 2-3 do not produce the same alteration of thalamic terminations and result in a pattern of projections similar to those observed in normal animals. These findings suggest that the presence of layer 4 strongly influences the normal distribution of thalamic afferent fibers and may modify the overall function of neocortex.

The use of the MAM

MAM is clearly identified as a useful tool to disrupt, or interfere with, the development of specific nuclear structures. Several groups demonstrate that administration of MAM during gestation results in selective neuronal loss, due to the prevention of DNA synthesis during a restricted window of time (Matsumoto et al., 1972; Evans and Jenkins, 1976; Noctor, 1998). MAM has also been reported to be selective for interfering with the proliferation of neurons but not astrocytes (Cattaneo et al., 1995). Although MAM is potentially carcinogenic,

such results occur with higher or prolonged use, and the single low dose administered in this study does not produce such effects (Zedeck and Swislocki, 1975; Zedeck and Brown, 1977). Using appropriately timed BRDU injections, we previously demonstrated that DNA synthesis resumes within 24 hours after MAM injection and that relatively normal birth of cells and migration of neurons resumes after MAM treatment (Noctor, 1998; Noctor et al., 1999). Other studies report resumption of normal cell birth after about 12 hours (Evans and Jenkins, 1976).

MAM injections have been used previously to selectively lesion layers of the cerebral cortex (Johnston and Coyle, 1979; Jones et al., 1982; Yurkewicz et al., 1984; Virgili et al., 1988; Woo and Finlay, 1996; Noctor et al., 1999). In an earlier study, we found that delivery of MAM according to the schedule described here results in discrete and specific disruption of either layer 4 in ferret somatosensory cortex (E33 delivery) or of layer 2-3 (E38 delivery) (Noctor, 1998). We have also added to these general findings by demonstrating that the overall size of the most significant thalamic nucleus projecting to the somatosensory cortex, the ventrobasal nucleus, is not altered. This suggests that the cells populating VB are largely born prior to MAM injection, as would be predicted from general knowledge of the birthdates of thalamic nuclei (Bayer and Altman, 1991). Although it is also possible that MAM treatment might interfere with the development of other systems that impact on the neocortex, our previous work, along with current observations on the thalamus, suggest that

most other subcortical nuclear groups potentially impacting on neocortical development are already born at the time of the MAM injections in this study. We also found that the effects of MAM were highly specific to the date of the injection. Administration of MAM on E38 did not produce the same layer deletion or alteration in the distribution of thalamic fibers. This implies that MAM acts through a narrow window of time leading to specific interruption of the cells born during that period.

Other researchers using MAM to interfere with the development of specific neocortical layers and to study subsequent projections of the thalamus find results slightly different from those reported here. Woo and Finlay (1996) determined that despite relatively complete destruction of layer 4 in the visual cortex of the hamster, the lateral geniculate nucleus projects a relatively normal bilaminar pattern upon the visual cortex, although the projection pattern is somewhat delayed. Jones et al. (1982) and Yurkewicz et al. (1984) also found a bilaminar distribution of thalamic projections in the somatomotor cortex of the rat after MAM induced disruption of layers 2-4, but determined that many of the thalamic terminals associated with layer 5 pyramidal cells. Jones and colleagues suggested that the thalamic terminals preferred sites to which they were normally attracted, whereas Woo and Finlay concluded that it was not necessary for the thalamic projections to terminate on specific neurons, but could distribute to almost any available cell. Both groups, however, found the terminations in a distinct bilaminar pattern.

In our experiment, the thalamic projections did not terminate in a distinct laminar pattern, but were almost equally distributed through all cortical layers. This suggests that cells other than those belonging to layer 4 can also serve as targets for thalamocortical afferent fibers. The earlier studies also suggest that other sites may serve as targets, since layer 4 was largely missing, but the observed thalamic terminations occurring in close proximity to their normal terminal site. It is possible that the different species used (our study used ferrets, whereas the experiments described above used rats or hamsters) might result in different terminal patterns of thalamic afferents. Since the ferret has a more protracted gestation than rats or hamsters, MAM treatment may more specifically and differentially eliminate a cortical layer depending on the time of injection. In the rat model, it may be more difficult to eliminate a specific layer because the gestation period is shorter.

For either case, in the absence of the preferred layer 4 target cells, the thalamic fibers terminate on the least objectionable destination. In normal animals, although layer 4 is the primary aim of thalamic afferents (and layer 6 in certain cytoarchitectonic fields), other layers also receive thalamic fibers in a lesser quantity. Additionally, outside of primary sensory areas, other layers receive a larger percentage of thalamic projections (Jones and Burton, 1976). So although thalamic afferents clearly prefer layer 4, it may be possible for them to terminate on cells other than layer 4 even in normal circumstances.

What is the nature of neocortical influence on thalamic termination?

There is some evidence that thalamic fibers operate under a degree of autonomy as they grow toward the neocortex, suggesting that at least the initial axonal outgrowth may occur without cortical cues (Molnar et al., 1999). Despite this initial independence, many studies support the idea that the neocortex exerts a trophic effect on axons leaving the thalamus. For example, *in vitro* studies evaluating conditions of axonal growth from the thalamus report that pieces of thalamus grown independently emit many fewer fibers than when cultured slices grow in the proximity of cortical pieces (Molnar and Blakemore, 1999). Also in support of the idea that the cortex can exert a trophic effect are the observations that specific molecules located within the cortex itself have been identified as attractive to thalamic axons (Mann et al., 1998; Barbe and Levitt, 1992). The same molecule can also be repulsive, however, depending on the nature of the afferent projection and the maturity of the structures involved (Mann et al., 1998; Barbe and Levitt, 1992).

On the other hand, several molecules, not specifically located in the neocortex, have been identified as significant in thalamic axon pathfinding, or in regional attraction or repulsion of thalamic axons. In mice missing the gene for Pax6, axons leave the thalamus fail to ascend toward the cortex. and grow in aberrant patterns away from their normal cortical targets (Kawano et al., 1999).

Whether or not details of the topographic arrangements and thalamic arborization refine under neocortical control are subjects of intense scrutiny.

Many studies support the idea that details of topographic projections and thalamic arborization are strongly influenced by features of the neocortex. The presence of intrinsic neocortical activity is a likely candidate for influencing thalamic refinement. Dating over several decades, studies involving the visual system, beginning with the work of Hubel and Wiesel, find that silencing or limiting activity in the cortex results in aberrant thalamic terminations, suggesting that the intrinsic activity of the cortex strongly influences the detail of thalamic termination (Hubel and Wiesel, 1977; Stryker and Harris, 1986; Katz and Shatz, 1996; Antonini and Stryker, 1996).

What is the nature of layer 4 influence?

There are two obvious functions that layer 4 cells might carry out during thalamocortical growth: they might attract ingrowing afferents and/or they might instruct afferent fibers to stop. If layer 4 cells are important for attracting or guiding thalamic axons, one would predict disturbance of thalamocortical projection in the relative absence of layer 4. Our observations, however, demonstrate that thalamic afferents project directly to the appropriate region of cortex in E33 MAM-treated animals. Ongoing studies in our lab also find that topography is preserved in ferrets receiving MAM injections that interfere with layer 4 production, indicating that relatively precise positional relationships are preserved in the thalamic projections (McLaughlin and Juliano, 1998). These observations suggest that although the influence of the cortex may be important for thalamic attraction, layer 4 is not required for this process.

Nevertheless, many authors conclude that layer 4 provides important cues instructing thalamic afferent fibers where to terminate or "stop". Several groups independently conclude that thalamic afferents will not grow into the neocortex until a specific level of maturity is achieved, which presumably coincides with the arrival of layer 4 (Molnar et al., 1999; Molnar and Blakemore, 1999; Molnar and Blakemore, 1995; Bolz, 1994; O'Leary et al., 1994; Gotz et al., 1992). *In vitro* studies of cortical slices cultured with pieces of thalamus report that axons leaving the thalamus find and terminate in layer 4 regardless of whether the thalamic piece is juxtaposed to the pial or subcortical surface (Gotz, et al, 1992; Yamamoto et al., 1997; Molnar and Blakemore, 1999). Other studies of organotypic cocultures report that growing thalamic axons appear to recognize layer 4 where they stop, branch, and display growth cone collapse. If the axons enter the cortical piece laterally, rather than from the pial or white matter surface, however, the stopping behavior was not observed (Yamamoto et al., 1997). This suggests that axons encountering layer 4 from a different cortical "zone" find a specific molecular signal that instructs them to stop and to branch. Even in the absence of these explicit signals in the E33 MAM-treated animals, afferent fibers eventually stop and branch suggesting that even without specific layer 4 cues, arbors are capable of forming. This implies that other mechanisms participate in allowing thalamic arbors to form, and may include some of the features likely to play a role in producing topographic precision, such as the activity of cortical cells, whether extrinsically or intrinsically derived.

How does diminution of layer 4 affect function?

Our preliminary results indicate that overall topography is preserved within the somatosensory cortex of E33 MAM-treated animals (McLaughlin and Juliano, 1998). In addition, although not tested behaviorally, the ferrets appear typical in their activities (McLaughlin and Juliano, unpublished observations). Despite this superficial normality, electrophysiological recordings in the MAM-treated animals find an altered laminar distribution of activity using current source density profiles. In the E33 MAM-treated somatosensory cortex, rather than initial sinks of activity observed in layer 4, the incoming activity was equally distributed throughout all layers, mimicking the distribution of thalamic afferents (McLaughlin and Juliano, 1999). Additional recordings detect that frequency following capabilities in E33 MAM-treated somatosensory cortex are impaired compared to normal animals (McLaughlin and Juliano, 1999). These observations, although preliminary, imply that the cortical responses in animals that lack most of layer 4 are modified in a manner that reflects the altered distribution of thalamic fibers. The question remains as to whether the thalamic fibers confer functional changes, or whether a quality intrinsic to the cortex influences or instructs the thalamic distribution.

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Figure Legends

Figure 1. An example of an injection site in a coronal section taken through the ventrobasal (VB) thalamus of a P14 ferret. The center of the DiI injection site is indicated with an asterisk; fibers can be seen emanating toward the somatosensory cortex. VB is delineated with a dashed line. Scale equals 1 mm.

Figure 2. Drawings of ferret brains from the dorsal perspective at P1 (A), P7 (B), and P14 (C). The region used for quantitative analysis is indicated with a shaded bar located just posterior and lateral to the post cruciate dimple (asterisk). At P1, very few sulci are visible, sulci become more evident at P7 and P14. Cru S, cruciate sulcus; Cor S, coronal sulcus; Ans S, ansate sulcus; Lat S, lateral sulcus. Scale equals 10 mm.

Figure 3. DiI label resulting from an injection into the thalamus of a normal animal at E33. A dense band of labeled fibers in the intermediate zone (IZ) emanating from the thalamus can be seen immediately superior to the ventricular zone (VZ). Fibers extend toward, but do not enter the cortex. Occasional retrogradely labeled cells in the subplate (SP) can also be seen. The cortical plate (CP) is very thin and the subplate is of substantial size. Scale equals 100 μm.

Figure 4. Examples of normal somatosensory cortex at P1, P7, and P14 stained with bisbenzimide. At P1, the cortical layers are not easily distinguished; observable are layer 1, the undifferentiated cortical plate (CP), and the poorly

distinguished layers 5 and 6. At P7, layers 5 and 6 are more easily delineated, and the upper part of the undifferentiated cortical plate is beginning to be distinguished from the lower part of the cortical plate. At P14, the cortical layers are more easily identified. The cortical plate can easily be separated into upper and lower tiers, and layers 5 and 6 are clearly discernable. Scale equals 500 μm.

Figure 5. Graph denoting the thickness of somatosensory cortex in normal and MAM-treated animals at different ages. At P1, cortical thickness is similar in all groups; the E33 MAM-treated cortex is slightly thinner, but this value is not significantly different from normal. At P7, the cortex is thicker, but there are still no significant differences between MAM-treated and normal. By P14, the E33 MAM-treated cortex is significantly thinner than normal (asterisk, two-tailed t-test, $p < 0.004$)

Figure 6. DiI label in the somatosensory cortex of normal and MAM-treated animals after injections in VB thalamus. At P1 (upper row) labeled fibers can be seen primarily in the deeper aspects of the cortex (immature layers 5 and 6). At P7 (middle row) distinctions between the thalamic terminations after MAM-treatment can be observed, with more fibers ending toward the upper portion of the cortex after E33 MAM-treatment, compared with either normal or E38 MAM treated cortex. At P14 (lower row) the thalamic terminations are almost equally distributed through all cortical layers in the E33 MAM-treated brains, this is in contrast to the label observed in the normal and E38 MAM-treated animals,

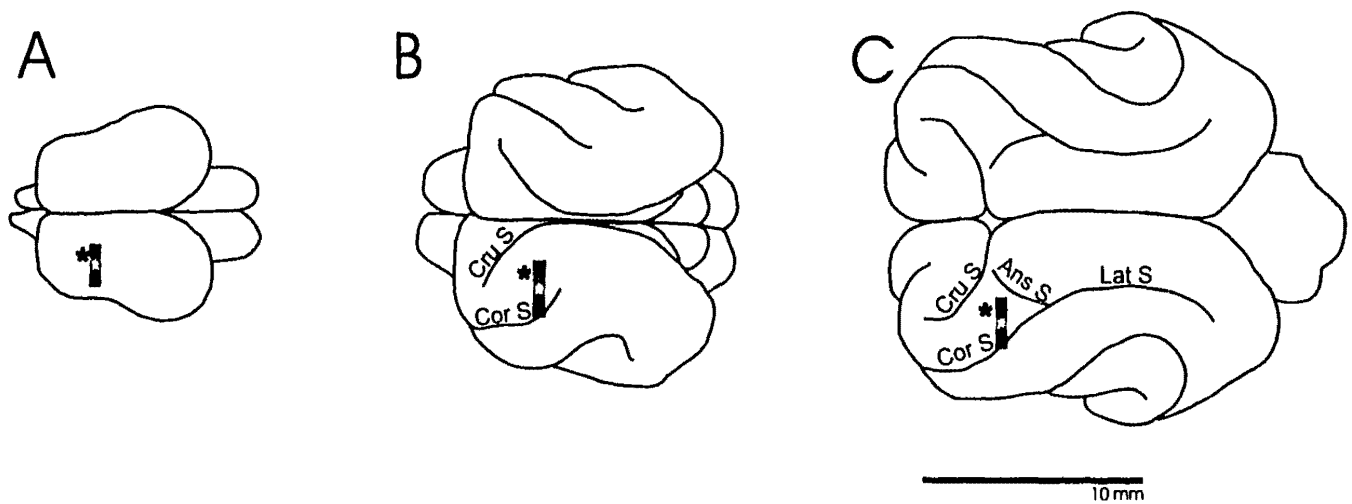
which contain thalamic terminations more focussed toward the central and lower layers.

Figure 7. Graphs indicating the distribution of thalamocortical fibers in the cerebral cortex at P1 in Normal, E33, and E38 MAM-treated somatosensory cortex. At this age, the following layers could be distinguished: layer 1, the undifferentiated cortical plate (CP), and layers 5 and 6. The values in each layer are expressed as a percent of the total fibers found in all cortical layers. There were no significant differences between any of the groups compared to normal (Mann-Whitney U Test).

Figure 8. Graphs indicating the distribution of thalamocortical fibers in the cerebral cortex at P7 in Normal, E33, and E38 MAM-treated somatosensory cortex. At this age, the following layers could be distinguished: layer 1, the undifferentiated cortical plate (CP), and layers 5 and 6. The values in each layer are expressed as a percent of the total fibers found in all cortical layers. The amount of thalamic fibers in layer 1 ($p=0.012$) and the CP ($p=0.048$) were significantly greater in the E33 MAM-treated animals compared to normal (asterisks).

Figure 9. Graphs indicating the distribution of thalamocortical fibers in the cerebral cortex at P14 in Normal, E33, and E38 MAM-treated somatosensory cortex. At this age, the following layers could be distinguished: layer 1, the upper part of the cortical plate (UCP, presumptive layers 2-3), the lower part of the cortical plate (LCP, presumptive layer 4), layers 5 and 6. The values in each

layer are expressed as a percent of the total fibers found in all cortical layers. The amount of thalamic fibers in layer 1 ($p=0.05$), the UCP ($p=0.021$), and layer 5 ($p=0.009$) were significantly greater in the E33 MAM-treated animals compared to normal (asterisk).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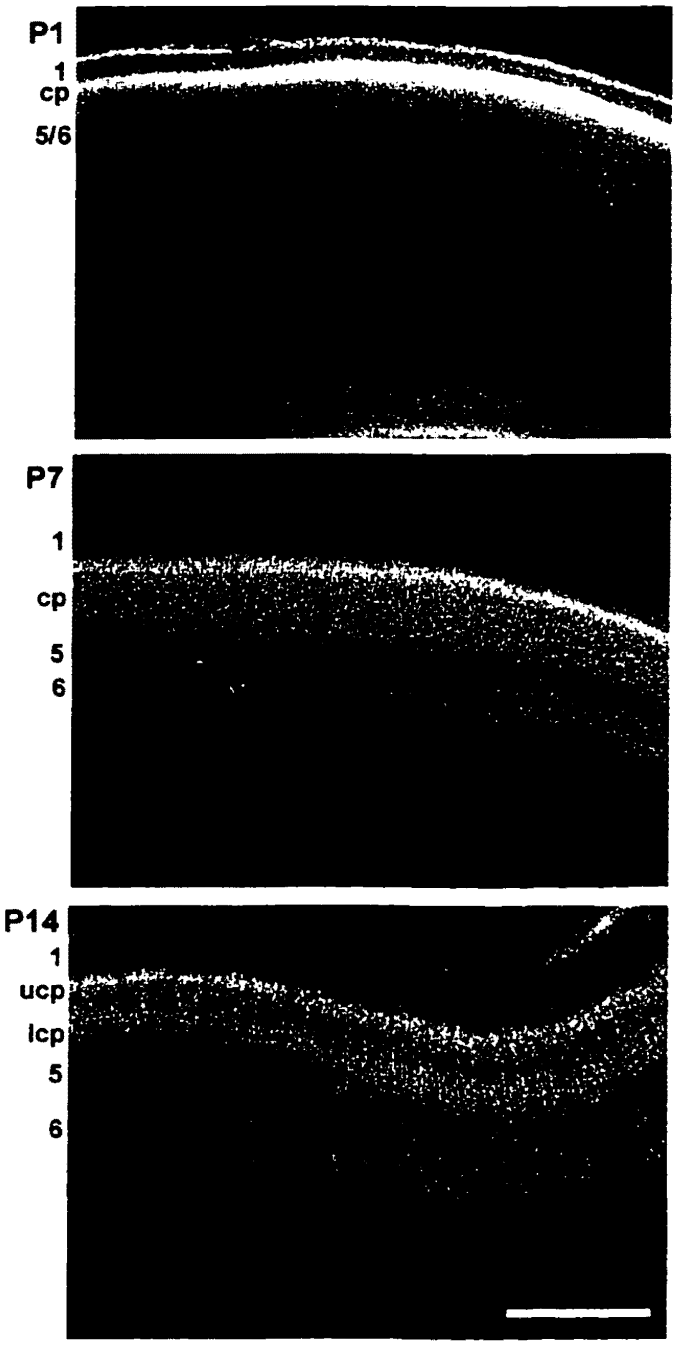
P1
1
cp
5/6
P7
1
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5
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P14
1
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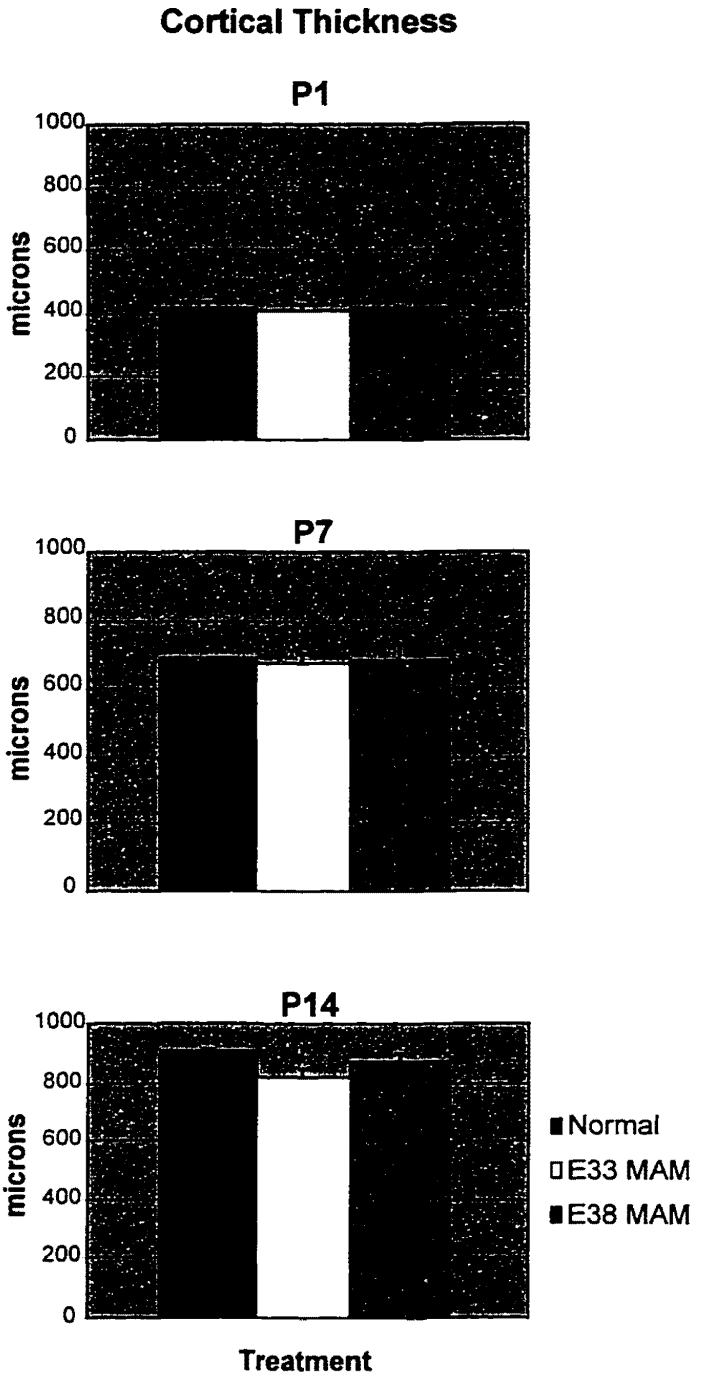
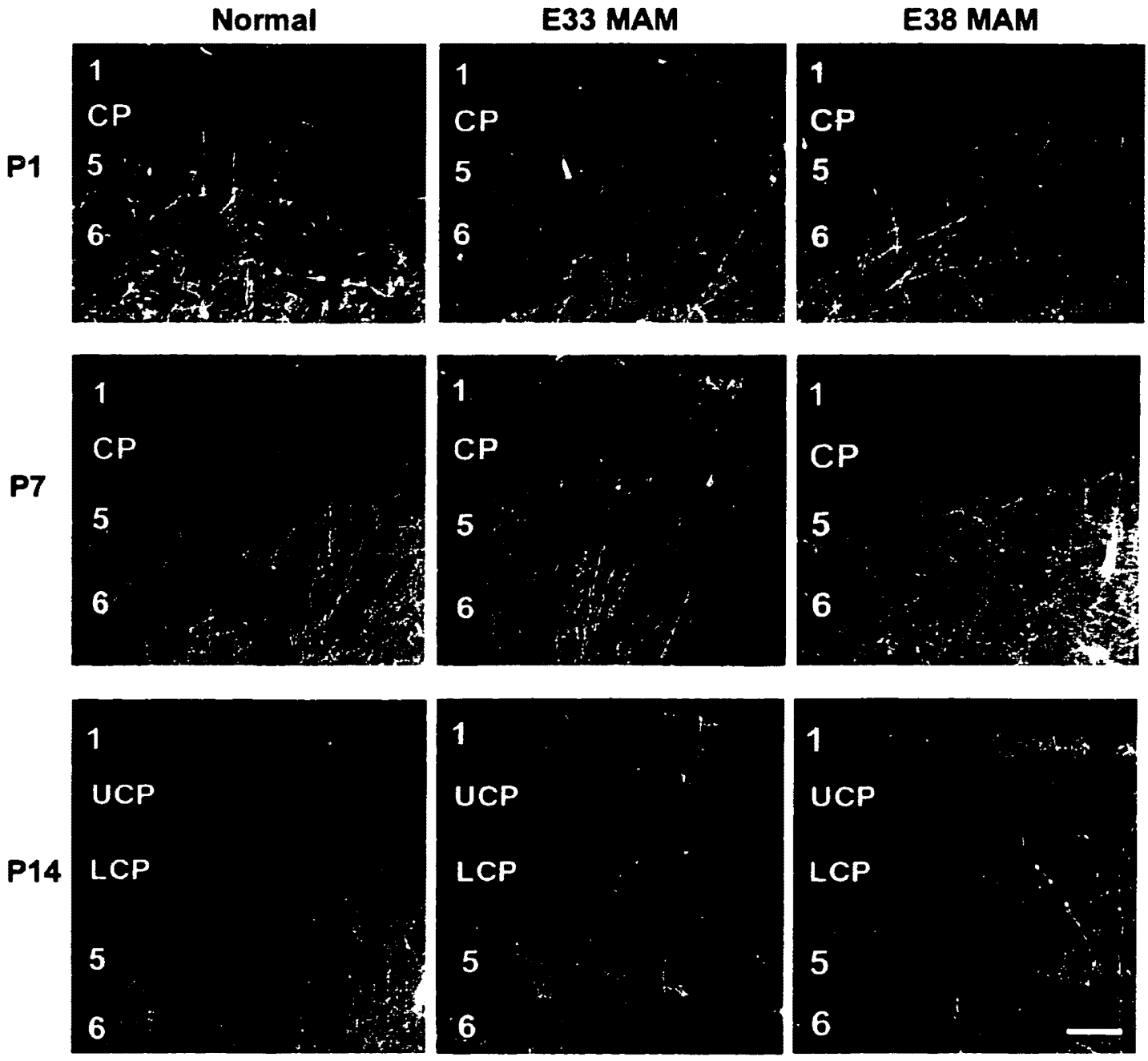

Cortical Thickness
P1
P7
P14
microns
1000
800
600
400
200
0
Treatment
Normal
E33 MAM
E38 MAM


Normal
E33 MAM
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P1
P7
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1
CP
5
6
1
UCP
LCP
5
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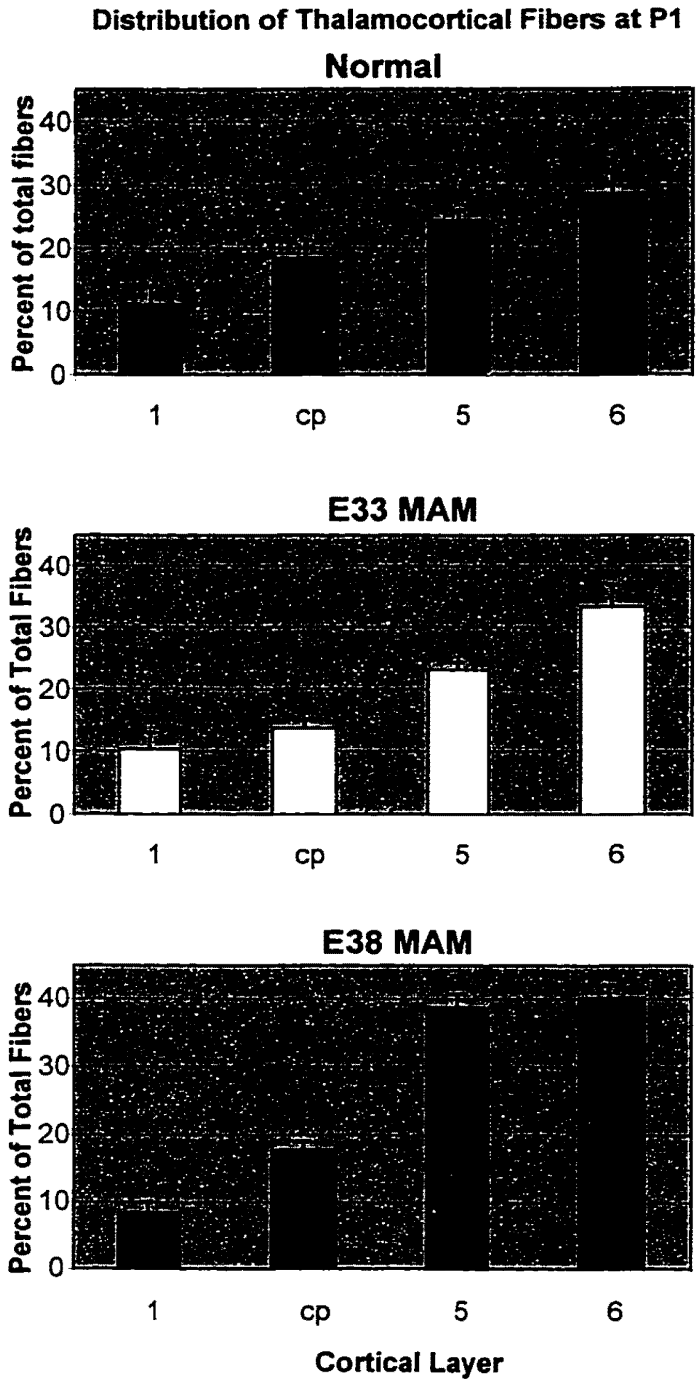
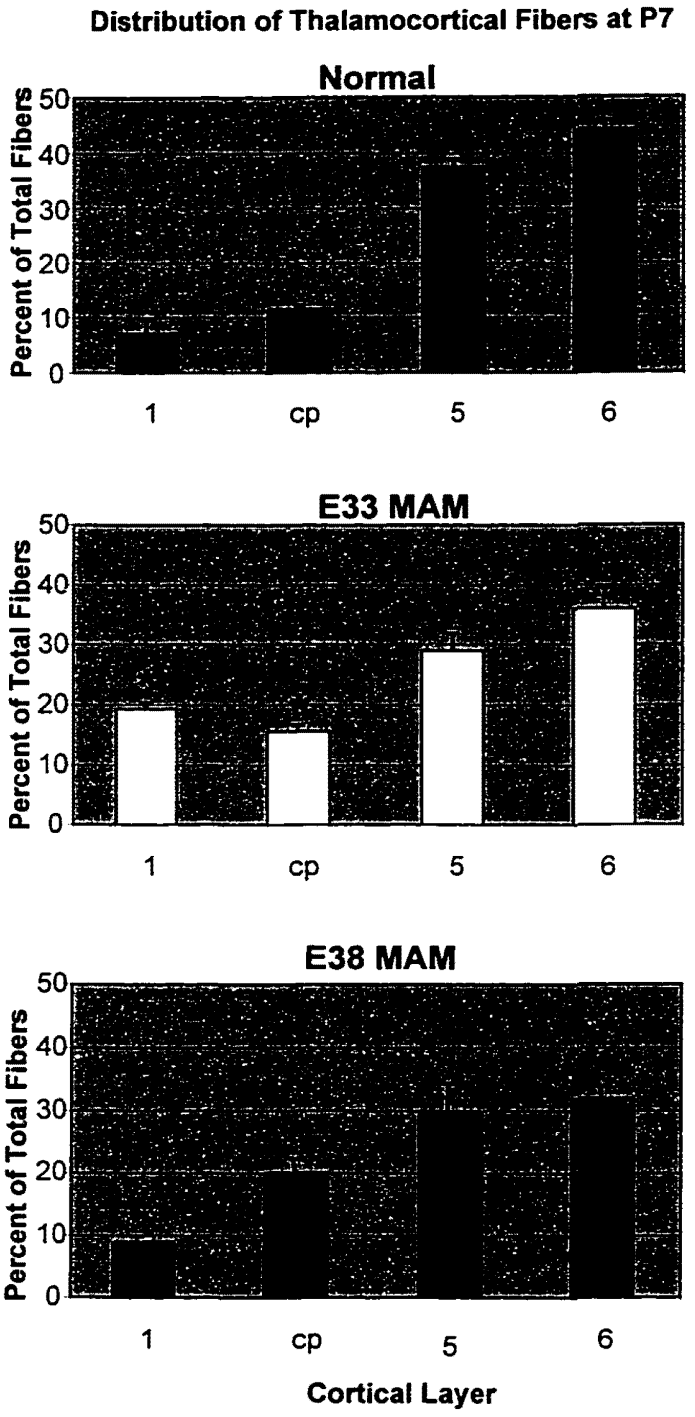

Distribution of Thalamocortical Fibers at P1
Normal
Percent of total fibers
0
10
20
30
40
1
cp
5
6
E33 MAM
Percent of Total Fibers
0
10
20
30
40
1
cp
5
6
E38 MAM
Percent of Total Fibers
0
10
20
30
40
1
cp
5
6
Cortical Layer


Distribution of Thalamocortical Fibers at P7
Normal
Percent of Total Fibers
0
10
20
30
40
50
1
cp
5
6
E33 MAM
Percent of Total Fibers
0
10
20
30
40
50
1
cp
5
6
E38 MAM
Percent of Total Fibers
0
10
20
30
40
50
1
cp
5
6
Cortical Layer

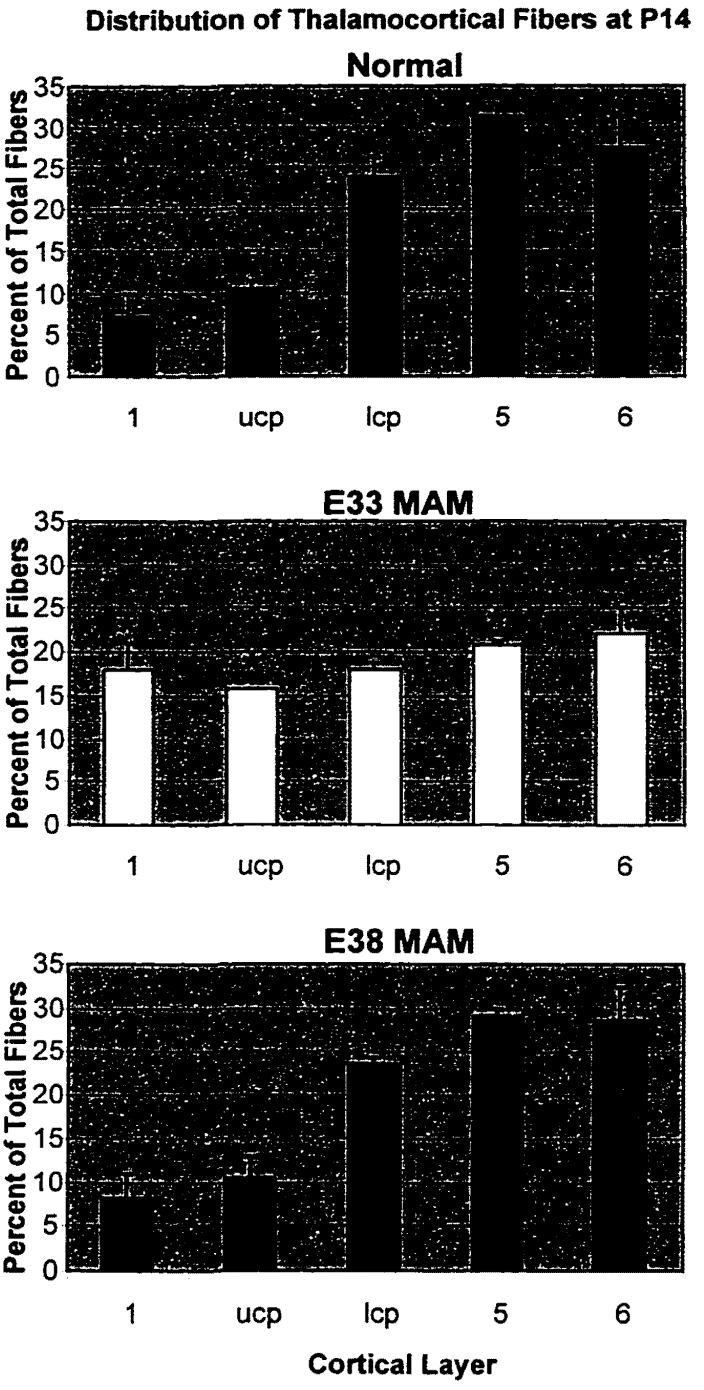

Distribution of Thalamocortical Fibers at P14
Normal
Percent of Total Fibers
0
5
10
15
20
25
30
35
1
ucp
lcp
5
6
E33 MAM
Percent of Total Fibers
0
5
10
15
20
25
30
35
1
ucp
lcp
5
6
E38 MAM
Percent of Total Fibers
0
5
10
15
20
25
30
35
1
ucp
lcp
5
6
Cortical Layer

TABLE 1

Number of pregnant ferrets

Normal	7
E33 MAM	7
E38 MAM	3

Number of kits analyzed for each age and treatment

	E33	P1	P7	P14
E33 MAM		5	4	3
E38 MAM		2	3	2
Normal	3	5	3	3

TABLE 2

Volume of anterior 500μm of ventral basal thalamus in mm^3

	Normal	E33 MAM
P1	0.1267	0.1277
P7	0.1754	0.1805
P14	0.2049	0.1981

Values represent means obtained from three animals at each age and treatment. Differences between normal and treatment at each age were not statistically significant.

LAMINAR SPECIFIC ALTERATIONS OF THALAMOCORTICAL PROJECTIONS IN ORGANOTYPIC CULTURES FOLLOWING LAYER 4 DISRUPTION IN FERRET SOMATOSENSORY CORTEX

Sidney L. Palmer, Stephen C. Noctor, Sharon L. Juliano

Department of Anatomy & Cell Biology, USUHS, Bethesda, MD 20814

Abstract

The developing neocortex appears to exert a number of influences on the development of the precise projections from the dorsal thalamus. Identifying these factors and the role they play in organizing thalamic input to the cortex and their overall role in shaping cortical organization has become an area of intense research interest. Within the cortex, layer 4 receives the majority of input from the thalamus and has been identified by a number of researchers as being important for instructing thalamic afferents to terminate. Previous *in vivo* experiments performed by this lab have demonstrated that following disruption to layer 4 formation in ferret somatosensory cortex by application of the drug methylazoxymethanol acetate (MAM), thalamic afferents fail to terminate in appropriate cortical regions. Thalamic afferents did however terminate correctly in cortex in which MAM application disrupted non-thalamic target layer 2. To further explore the role of layer 4 in thalamocortical development, we prepared organotypic cocultures consisting of normal P0 ferret thalamus paired with normal, E33 or E38 MAM-treated cortex obtained from ferrets at either P0 or P7. MAM injection on E33 disrupts layer 4 formation whereas a similar injection on E38 has been shown to interfere with layer 2 formation. The cocultures were allowed to grow together for a number of days in culture, then a discrete injection of either fluorescent dextrans or the carbocyanine dye, DiI were made into the thalamic piece. The labeled thalamic afferents that grew into the cortical slice

were then analyzed and the sites of their terminations were quantified after 3, 5, or 7-10 days in culture (dic). After 3dic, significant differences were noted. In normal cocultures, the largest percentage of fibers terminated within appropriate cortical regions. Thalamic fibers invading both P0 and P7, E33 MAM-treated cortex failed to terminate significantly within the developing cortical plate. Thalamic ingrowth into E38 MAM treated cortex revealed a pattern of termination similar to normal. By 5dic, not only did thalamic afferents terminate within the developing cortical plate region of both P0 and P7 cocultures, but they also demonstrated more mature looking terminations and branching patterns. Thalamic afferents growing into E33 MAM-treated cortex grew beyond the cortical plate and many fibers were found terminating inappropriately within lower cortical layers or white matter. By 7-10dic, the overall pattern of terminations was not altered. The preponderance of thalamic fibers in normal P0 or P7 cortex were found terminating in the region of layer 4 whereas their counterparts in E33 MAM-treated cortex were found to terminate in other regions. In all cases, terminal distribution of thalamic fibers in E38 MAM-treated cortex looked similar to normal. These results demonstrate the role of layer 4 as providing thalamic afferents with important positional and termination cues as well as adding credence to the previous *in vivo* data demonstrating similar findings.

Introduction

The mammalian neocortex consists of numerous areas differing in connectivity and functional properties; all of these areas have highly defined interrelationships with thalamic nuclei. In addition to a distinct relationship between thalamic nuclei and corresponding cortical areas, thalamic afferents target specific cortical layers as sites of termination. In primary sensory areas of cortex, thalamic afferents arborize and terminate predominantly within layer 4 (Jones and Burton, 1976). The mechanism that controls the specificity of terminations is not fully understood, although researchers observing the growth of thalamic afferents into developing cortex suggest that layer 4 may possess a "stop", or recognition, signal. This recognition cue may instruct extending thalamic afferents to arborize and form connections within the appropriate target layer (Molnar and Blakemore, 1995; Molnar et al., 1998; Molnar and Blakemore, 1999).

To specifically assess the role of layer 4 in directing proper thalamic terminations, we developed an *in vitro* model to study thalamic growth and termination into slices of cerebral cortex in which the development of layer 4 was disrupted. We interfered with layer 4 development in ferret somatosensory cortex by injections of methylazoxy methanol (MAM) into pregnant ferrets on appropriate gestational days. MAM is an antimitotic that prevents cells from dividing for a short period, thereby hindering the birth of a given population of

cells that would normally populate a specific layer of cerebral cortex (Matsumoto and Higa, 1966; Zedeck et al., 1970; Matsumoto et al., 1972; Johnston et al., 1979; Jones et al., 1982; Cattabeni and Di Luca, 1997). Injections of MAM into ferrets on the appropriate gestational day clearly result in a dramatic decrease in the dimension and constituents of layer 4 in somatosensory cortex, with little other obvious effects in neocortical development (Noctor, 1998; Noctor et al., 1999).

In the present study we examine the effect of pairing MAM-treated cortex with slices of normal ventrobasal thalamus (VB) in the organotypic coculture environment. We previously established that MAM administration on embryonic day 33 (E33) results in substantial reduction of the constituents of layer 4 in ferret somatosensory cortex (Noctor, 1998). The disruption of layer 4, as the primary site of thalamic termination, is likely to strongly influence the distribution of afferents from the thalamus. Injections of MAM on E38, on the other hand, will interrupt the generation of layer 3-2 of ferret somatosensory cortex, and therefore should have little effect on the terminal pattern of thalamic afferents. We elected to study thalamocortical development using an *in vitro* model to allow for control of detail and specifics of the environment. We previously established, using *in vivo* DiI injections, that the projections from VB into ferret somatosensory cortex were altered from the normal distribution in E33 MAM-treated ferrets, but not in those treated with MAM on E38. In the E33 MAM-treated cortex, the thalamic afferent fibers were almost equally distributed

through all cortical layers, rather than being focussed in the central layers, as in normal and E38 MAM-treated animals (Palmer, 1999). This finding suggests that a factor present in layer 4 instructs growing thalamic axons to stop in the vicinity of layer 4 and extend arbors. In the relative absence of layer 4 cells and the presumptive "stop" cue, growing thalamic fibers terminated non-specifically. The current *in vitro* study allows us to analyze the relationships between VB and somatosensory cortex in normal and MAM-treated animals in more detail than possible in the previous DiI studies. In addition, the arrangement of pairing organotypic cultures of normal thalamus with MAM-treated cortex allows us to rule out the possibility that the abnormal thalamic terminations result from a disruption in the thalamus itself after MAM treatment, rather than the specific diminution of layer 4. Using organotypic cocultures also allow us greater control over the positioning of small tracer injections and the ability to assess the thalamic terminations in detail, compared to the previous study.

Methods

Organotypic Cocultures

P0 ferret kits were deeply anesthetized with an IP injection of 50mg/kg Pentobarbital Na and their brains removed with quick dissection under aseptic conditions in ice cold artificial cerebral spinal fluid (aCSF). The aCSF contained (in mM): NaCl 124, $NaHCO_3$, NaH_2PO_4 1.2, KCl 3.2, $MgSO_4$ 1.2, $CaCl_2$ 2.4, glucose 10. Coronal slices of approximately 400 μm thick somatosensory cortex were made from each hemisphere using a tissue chopper. These slices were then placed in cell strainers (Falcon cell strainer, 70μm nylon mesh, Fisher) in culture wells (Corning cell wells, Fisher), covered with sufficient media until a meniscus of fluid covered the tissue, and placed in an incubator (5% CO_2/95% O_2). These cortical slices were allowed to settle and adhere from 1 hour to 1 day. At this point, thalamus from anesthetized P0 ferrets were dissected and regions corresponding to ventrobasal nuclei were removed under microscopic dissection. These thalamic pieces were then juxtaposed to the previously established cortical slice. In some cases, the thalamic piece abutted the pial surface and in other cases, they were adjacent to the white matter. We used Minimal Essential Medium (MEM) with Earles Salts; also added were 10 mg gentamycin, 10 ml glutamine, 6 g glucose (per 1000 ml of media), and 10% heat inactivated normal horse serum. After 2 days in culture, antimitotics were added to the media to prevent excessive growth of glial cells including: 5-fluoro-2-deoxyuridine, cystine

beta-D-arabanofuranofuranoside (1 mg each in 4.1 ml media, to make a stock solution). Stock solution was added to MEM to make a 4.4 μM final concentration of antimitotic media.

We used the presumptive somatosensory cortex to prepare the organotypic cultures of cortex and the ventrobasal nucleus for the thalamic piece. Although the somatosensory cortex cannot be definitively identified at P0 or P7, we could locate the region of the somatosensory cortex fairly accurately using landmarks that are present, even at birth, especially the post cruciate dimple (Juliano et al., 1996; Noctor et al., 1999; Palmer, 1999). Additionally, the ventrobasal thalamus was located using horizontal sections that we cut to use as a guide for locating specific structures. Using the horizontal bisbenzimide stained-sections we could identify the precise rostro-caudal location of VB to select the appropriate slices for coculture with the cortical pieces.

For one set of organotypic cultures, cortical slices were analyzed independently to determine the viability and maturation of the cultures over time *in vitro*. To do this, we made iontophoretic injections of dextrans (as described above) in various positions in the culture, and used bisbenzimide staining to evaluate cortical structure and lamination.

MAM Treatment and Disruption of Layer Formation

Pregnant ferrets were anesthetized with 5% halothane and 0.05% N_2 O. An injection of MAM (12mg/kg, dissolved in saline) was administered IP on E33

or E38. MAM injections on E33 prevent a large portion of layer 4 from being formed, while administration of MAM on E38 disrupts the formation of layers 3-2, a non-target layer for thalamic afferents (Noctor et al., 1997).

Anatomical Tracer Injections

The cocultured slices were removed from the incubator at 3, 5, 7, or 10 DIC. At this point, the cocultures were transferred to an oxygenated slice chamber perfused continuously with aCSF and the thalamic pieces injected with one of two types of anatomic tracer. Some of the slices received injections of dextrans (10,000 MW, Fluroruby, Molecular Probes, Eugene, OR), which were placed in several locations iontophoretically (+alternating current, 6 μA, 3-4 minutes) within the thalamic piece using a pipette (tip diameter 15-20 μm). Following the injections, the slices were maintained in the chamber for up to 8 hours to allow for transport of the tracer. In some of the cultures, fluorescent-tagged dextran injections were made in specific sites of the cerebral cortex to determine if the cortical slices maintained features that characterize ferret somatosensory cortex during development (Juliano et al., 1996). In some of the cocultures a small crystal of the lipophilic dye, 1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate (DiI) was placed in the thalamic piece using pipette. The cocultured slices were then returned to the incubator and the tracer allowed to transport for up to 12 hours. All cocultures labeled as described above were then removed from the incubator and fixed in 4% paraformaldehyde

in 0.1 M phosphate buffer overnight and counterstained with the nuclear marker, bisbenzimide, prior to mounting on gelatin-subbed glass slides.

Analysis

Organotypic cocultures were analyzed on a fluorescent microscope outfitted with a CCD camera, which allowed us to capture the images onto a computer for enhancement and evaluation. The data from the injections was drawn using the software package Neurolucida, then the fibers and terminations were analyzed and quantified. For puposes of analysis, we termed the end of a fiber as a termination. Using the bisbenzimide label for each coculture, we partitioned the cortical slice into circumferential bins beginning at the pial surface, of 100 μm depth each, for 6 bins. We then counted the number of fiber terminations found in each bin. The fiber terminations in each bin were then expressed as a percentage of total fibers to allow us to compare between differences in the injection sizes. For purposes of convenience in the analysis, after locating each terminal fiber within a 100 μm bin, crude cortical layers were assigned to each depth, using the bisbenzimide counterstain. Although layers could usually be identified quite clearly, despite up to 10 days in culture, the following distinctions were assigned to the cortical cultures: layer 1 (which usually corresponded to the first 100 μm deep from the pial surface), the cortical plate (CP, which varied in size and depth depending on the age of the cortical culture and the days in culture), layers 5 and 6 (which also varied in size and

relative position depending on the age of the slice and time in culture).

Results

Eighteen pregnant ferrets were used for this study. Ten of the ferrets were normal, 5 received an injection of MAM on E33, and 3 received MAM injections on E38. Forty-seven kits were used to obtain 143 slice pairs for organotypic cocultures (Table 1). Most of the cultures were made on P0, for both the thalamus and the cortex pieces. In a few cases, P0 thalamus was paired with P7 cortex, to determine if the age of the cortex influenced ingrowth of thalamic afferents (Table 1). Cortical slices from normal, E33, and E38 MAM-treated ferrets at P0 or P7 were paired with normal thalamus also obtained from ferrets at P0. Thalamic pieces were juxtaposed with the cortical slices either directly against the pial surface or against the white matter/ventricular zone. Several researchers have demonstrated that the thalamus extends normal appearing arbors specifically into layer 4 in organotypic cocultures, despite positioning of the thalamic piece adjacent to the pia or adjacent to the white matter (Bolz et al., 1990; Bolz et al., 1992; Molnar and Blakemore, 1999) . In fact, we found that positioning the thalamic piece adjacent to the pia was a highly successful technique, likely because the distance required for the thalamic axons to grow was reduced compared to placement against the white matter.

Characteristics of normal cortical organotypic cultures

The normal maturation of cortical organotypic cultures was studied for up to 10 days. We evaluated the laminar pattern, as well as specific features of

cortical architecture after different times in culture; both were compared to normal acute slices. We accomplished this using bisbenzimide staining and strategically placed injections of dextrans. The bisbenzimide staining demonstrates that the laminar architecture undergoes maturation and appears relatively normal. Shown in Figure 1 are examples of bisbenzimide staining in cortical slices, after various times in culture. The cytoarchitecture shows clear evidence of a distinct laminar pattern at all ages evaluated. In an acute slice, the dense cortical plate is obvious, as is the poorly differentiated layers 5 and 6 (Figure 1A). After 3 days in culture, increased evidence of laminar differentiation can be seen (Figure 1B). After 10 days in culture, the cortical layers are distinct and appear similar to the normal architecture at that age (Figure 1C).

Small injections of dextrans into the cortical cultures at different ages also revealed that other aspects of structural development proceed relatively normally in these cultures. Shown in Figure 2 are examples of small injections into cultures that were maintained for 6 or 10 days *in vitro*. The injections at the earlier time (6 days in culture) reveal a characteristic feature of immature cerebral cortex, the radial glial scaffold. We previously demonstrated that small injections of dextrans into acute slices of ferret somatosensory cortex, up to about 1 week of age, definitively label radial glia extending from the ventricular zone to the pial surface; this occurs after injections into the cortical plate, intermediate zone, or ventricular zone (Juliano et al., 1996). This feature is maintained in cultured slices, up to 6 days *in vitro*. At later times, the cortical

cultures continue to maintain characteristics similar to acute slices (Juliano et al., 1996). In normal acute slices at P10-14 (not cultured, but maintained in a slice chamber), the dextran injections lose their radial character, and typify the cortical architecture. This can be seen in Figure 2 as well, which demonstrates an example of an injection into an organotypic cortical culture after 10 days. In this instance, seen at higher power, dextran was injected into the approximate layer 5, and largely labels pyramidal cells, with apical dendrites extending toward the cortical surface.

Several factors were used to consider the overall health and viability of the cocultures. (1) We verified the presence of a laminar pattern, as discerned with the bisbenzimide counterstain. If organotypic cultures are not healthy, the distinction of cortical layers is not evident. (2) If organotypic cultures are not robust, they do not actively transport anatomical tracers (Juliano et al., 1996). The presence of viable transported label, therefore, is an indicator of the positive status of the coculture. (3) Representative sets of cocultures were removed at days in culture corresponding to the times of tracer injections and rather than receiving injections into the thalamic piece, were immunostained for antibodies directed against MAP2, an early marker for neurons. The presence of MAP2 immunoreactivity indicates the presence of viable neurons at the time of removal from the culture environment.

Distribution of thalamic afferents

Three Days in Culture

P0 cortex paired with P0 thalamus

In experiments pairing normal P0 cortex with normal P0 thalamus, robust clusters of fibers extended from the thalamic explant into the host cortex after only 3 days in culture. Some of these thalamic fibers extend considerable distances, ranging from 500-1000 μm to terminate in the upper tiers of the host cortex (Figure 3). Quantitative analysis of all normal cocultures after 3 days *in vitro*, pairing P0 thalamus with P0 cortex, revealed that approximately 48% of all labeled fibers terminated within the 100-200 μm bin of the cortical slice, a distance roughly corresponding to the immature cortical plate (Figure 4). An additional 27% of terminations occurred in the outermost 100 μm bin, corresponding to layer 1. An example of the focussed terminal pattern in the normal cocultures can be seen in Figure 5.

As with the normal pairs of cocultures, the cultures consisting of P0 E33 MAM-treated cortex paired with normal P0 thalamus contained labeled fibers that extended from the thalamic piece long distances into the cortical slice. The labeled fibers extended from 200 – 1200μm in length. The cocultures containing E33 MAM-treated cortex demonstrated less specificity in the thalamic sites of termination than the normal cocultures. While many fibers were observed terminating in the upper 100μm of the cortex (36%) the remainder of fibers

appeared to terminate without focus throughout other regions of the cortex or in the white matter (Figures 3 & 4). Significantly fewer fibers terminated within the bin 200 μm below the pial surface, compared to the normal distribution (Mann-Whitney U test, p=0.038, bin 2).

After 3 days in culture, organotypic cocultures consisting of E38 MAM-treated cortex at P0 and normal P0 thalamus displayed characteristics similar to that of the normal cocultures. The labeled fibers emanating from the thalamus grew to lengths ranging from 100 μm to over 2000 μm. Thirty eight percent of the labeled thalamic fibers in the E38 MAM-treated cortex were in the bin 100-200 μm below the pial surface and additional 23% was in the bin corresponding to layer 1 (Figures 3 & 4).

P7 cortex paired with P0 thalamus

The cocultures that consisted of normal thalamus and normal cortex obtained at P7, demonstrated a pattern of termination of labeled fibers originating from the thalamic piece similar to that of normal cocultures with the cortical piece obtained at P0. After 3 days in culture, 70% of the labeled thalamic terminals distributed within the locus of the cortical plate, i.e., between 200-300 μm from the pia (Figure 5). Because the cortical piece is older, the dimensions are different from the P0 culture. Although the distances from the pia were slightly different in the cortical slice obtained at P7, the overall terminal pattern was similar to that seen in the normal cocultures obtained from P0 cortex and P0

thalamus, except for the increase in layer 1 terminations in the P0 cortical culture.

Experiments pairing P7 E33 MAM-treated cortex with normal thalamus elicited distributions with many features similar to the cocultures using P0 cortex. In both instances, the thalamic terminations are distributed roughly equally in the cortical layers (Figures 4 & 6). The cocultures using P7 MAM-treated cortex, however, lacked an increase in thalamic terminations in the outermost portion of the cortex, a region likely to correspond with layer 1.

Five Days in Culture

P0 cortex paired with normal P0 thalamus

After 5 days in culture, fibers growing from normal thalamus into normal cortex began to establish a more complex appearance. Many of the thalamic fibers after 5 days in culture assume a more branched appearance in their terminal location (Figure 3). Ingrowth to cortex was similar to that observed after 3 days in culture, approximately 42% of the labeled afferent fibers were located 100-200μm below the pial surface, i.e., corresponding to the location of the cortical plate (Figure 8). About 20% of all fiber terminations were located within the upper 100 μm of cortex.

Thalamic fibers from normal thalamus growing into E33 MAM-treated cortex after 5 days in culture were not as highly branched as the thalamic fibers growing into normal cortex at this same period (Figure 3). The fiber lengths

emerging from the thalamus ranged from 100 μm to over 1200 μm. In many instances when the thalamus was placed adjacent to the pial surface of the cortical slice, outgrowth advanced into layer 1 of the cortical explant and extended long distances within this layer without further invasion into deeper cortex, accounting for nearly 40% of the total fibers observed in the cocultures analyzed at this age (Figures 5 & 8). Twenty seven percent of the total fibers terminated at the depth of the developing cortical plate (approximately 200 μm below pial surface; Figure 8). There were significant differences in the thalamic terminal distribution depths between the E33 MAM-treated and normal cortical slices (Mann-Whitney U test, p=0.025 bin 2, p=0.01 bin 3). An example of 5 days in culture fiber outgrowth into E33 MAM treated cortex can be seen in Figure 9.

Experiments pairing E38 MAM-treated cortex with normal thalamus demonstrated a fiber distribution within the cortical explant similar to the control. Thirty three percent of the labeled thalamic afferents were found in the bin between 100- 200 μm from the cortical surface. Approximately 23% of the thalamic fibers terminated within the first 200 μm bin below the pial surface, i.e., the most closely correlated to layer 1 (Figure 8). The thalamic afferent fibers displayed a branching structure similar to those observed in coculture controls (Figure 3).

P7 cortex paired with normal P0 thalamus

After 5 days in culture, the distribution of thalamic afferents arising from

normal thalamus paired with normal cortex focuses primarily at the level of the cortical plate, 200-300 μm below the pial surface. (Figure 10). This overall distribution is similar to that of P0 normal cortex paired with P0 normal thalamus. There were fewer examples of the P7 cortical slices, so statistical analysis was not conducted on these cocultures.

In the pairing of E33 MAM-treated cortex taken at P7 with normal P0 thalamus, the terminal fibers arising from the thalamic pieces lacked the specificity found in the normal to normal pairing. The terminal pattern was irregular through the different layers of cortex (Figure 10).

Seven -Ten Days in Culture

P0 cortex paired with P0 normal thalamus

At this time point, thalamic fibers from normal thalamus entering normal cortex continue to possess the branching structure observed at earlier time points. That is, many afferent fibers labeled from the thalamic piece extended arbors into the cortical plate (Figures 3 & 11A). Approximately 60% of the fibers analyzed terminated within the region identified as the cortical plate; 27% of all fibers were found within 100 μm from the pia, probably corresponding to layer 1 (Figures 11B & 12).

The cocultures that paired E33 MAM-treated cortex with normal thalamus continued to lack the distinctive branching characteristics seen in the control pairs (Figures 3 &12). The labeled thalamic fibers are distributed throughout the

100 μm bins, with a relatively high percentage in layer 1 (Figures 5A & 6B). There are significant differences between several of the depths of analysis compared to normal (Mann-Whitney U test, p=0.014, bin 2; p=0.047, bin 4).

As with shorter times in culture, the thalamic growth into E38 MAM-treated cortex after 7-10 DIC shared significant characteristics with control cocultures. As in the normal cortical pieces, the labeled fibers were branched near their terminals (Figure 3). Approximately 55 % of all the labeled thalamic afferent terminals occurred at the level of the cortical plate and 27% were found coincident with layer 1.

P7 cortex paired with P0 thalamus

After 7-10 days in culture, the normal thalamus paired with normal cortex obtained at P7 resulted in afferent terminations that were similar to those seen in cocultures using P0 normal cortex. Fifty six percent of the labeled terminations occurred in the cortical plate; similar to the amount seen in the normal cocultures obtained at P0 (Figure 13).

In the cocultures that paired E33 MAM-treated cortex obtained at P7 with normal thalamus the afferent thalamic terminals were distributed relatively randomly through all cortical layers, with a greater concentration of fibers in the deeper layers (Figure 13).

Discussion

Summary

We assessed the role of layer 4 in determining proper thalamic terminations in a culture environment. Organotypic cultures consisting of ferret somatosensory cortex obtained at either P0 or P7 placed with ventrobasal thalamus obtained at P0 displayed normal patterns of maturation over a 10-day period. Neurons in the thalamic piece extended axons into the cultured cortex and terminated in laminar locations appropriate for the age of the culture. The age of the cortical culture (i.e., P0 or P7) made little difference in the distribution of thalamic afferents. Similar cultures were obtained from ferrets at P0 or P7, in which layer 4 of somatosensory cortex was disrupted by *in utero* injection of MAM on E33. In the layer 4-disrupted cocultures, we found that axons originating from the thalamic piece did not terminate or arborize in focussed regions of the cortical plate. When cultures were made using cortical slices from animals treated with MAM on E38, the thalamic terminations were similar to those in normal cultures. E38 MAM treatment disrupts layer 2-3 in ferret somatosensory cortex and should not interfere with thalamic terminations. These findings support the idea that the presence of layer 4 is important for producing a normal pattern of thalamic innervation.

Effect and specificity of MAM treatment

Although the consequences of MAM administration are systemic, and could theoretically effect multiple levels of the CNS, the injections in this study are timed late enough not to interfere with the development of most structures that strongly influence neocortical maturation (Bayer and Altman, 1991). Our previous studies find that appropriately timed administration of MAM can specifically diminish a single layer of ferret somatosensory cortex (Noctor, 1998). Although there are slight effects in other layers, the primary result of MAM treatment is diminution of layer 4. We also find that MAM injections on a different date than that targeting layer 4 (i.e., the E38 injections interfering with layer 2-3) does not result in effects overlapping with E33 MAM treatment. This pertains either to quantitative analysis of the cytoarchitecture, *in vivo* analysis of the thalamocortical connections, or to the *in vitro* results reported in this study (Noctor, 1998; Palmer, 1999). In addition, Palmer (1999) determined that E33 MAM treatment in ferrets does not alter the dimension of the ventrobasal nuclear complex, which is the primary thalamic nucleus conveying somatosensory information to the cerebral cortex.

The organotypic culture environment

Many other investigators have developed organotypic cultures of cerebral cortex (Bolz et al., 1990; Yamamoto et al., 1992; Emerling and Lander, 1994; Yamamoto et al., 1997; Molnar and Blakemore, 1999) . As in our study, most reports observe relatively normal cortical development for a period of time in the

culture environment. In organotypic cultures, cortical laminae mature, neurons continue their migration into appropriate laminar positions, and expression of many molecules continues along a reasonable time frame (Caeser et al., 1989; Gotz and Bolz, 1989; Bolz et al., 1990; Bolz et al., 1992; Behar et al., 1999; Molnar and Blakemore, 1999). The time that neocortex can mature relatively normally in the culture environment is limited, but our studies did not take place over a period so unduly protracted to not maintain a reasonable level of cortical maturation. At least a moderate level of cortical development occurs in our ferret model, since neocortical cultures demonstrate progressive features that occur in acute slices at the same relative ages (Juliano et al., 1996). For example, after 6 days in culture, small tracer injections label radial glia, which are normally present at that time in normal ferret cortex. Tracer injections in 10 day old cultures, however, no longer label distinct radial glia, but delineate pyramidal neurons, which are not present at earlier times, indicating a certain level of maturation.

We also observed similarities to earlier reports in our pairing of thalamus and cortex in organotypic cultures. Thalamic pieces easily extend axons into the cortical piece from multiple orientations, as has been reported by others (Bolz et al., 1992; Bolz, 1994; Emerling and Lander, 1994; Molnar and Blakemore, 1999). The cerebral cortex attracts thalamic growth in culture preferentially over other potential targets, although whether a precise matching between specific thalamic nucleus and the cortical target is required is not clear (Bolz, 1994; Molnar and

Blakemore, 1999). This indicates that a feature present in the cortex attracts thalamic afferents, which agrees with our findings. We find this general attraction to hold whether we used normal or MAM-treated cortical slices, suggesting that the attractive factor is not located in layer 4 but in another neocortical locus.

The influence of cortical maturation

Molnar and others suggest that the cortex must reach a certain age before thalamic fibers can enter, suggesting that thalamic afferents are actively inhibited prior to the appropriate time (Bolz et al.,1990; Molnar and Blakemore, 1999). Molnar also indicates that in the coculture situation, there is a relatively discrete window during which thalamic afferents terminate most effectively in cortical slices. In our study, we used cortical slices obtained either at P0 or P7. The most effective age to obtain thalamic ingrowth according to Molnar and colleagues is P3. We decided to include a series obtaining cortical cultures at P7, since we were concerned that despite the fact that thalamic afferents are known to enter the somatosensory cortex by P0 in ferret, the cortex might be too immature to foster appropriate thalamic arborization (Juliano et al., 1996; Palmer, 1999). In addition, we are aware that the maturation of the cortex clearly influences the distribution of thalamic afferents (Shatz and Luskin, 1986; Miller et al., 1993). In our study, both P7 and P0 cortical cultures foster ingrowth of thalamic axons, however, with very little substantive differences between the two. This is not completely surprising, since although Molnar and Blakemore (1999) report that the best growth of thalamic afferents in organotypic coculture occurs in mouse

cortex using cultures obtained at P3, presumably due to the presence of permissive factors, our first date of analysis was after 3 days in culture, which would roughly correspond to the appropriate age. In addition, the maturation of ferret somatosensory cortex, does not necessarily correspond to the same window of time appropriate for mouse cortex. In fact, our previous *in vivo* data suggest that ferret somatosensory cortex accepts thalamic afferents at P0, and that they are roughly localized to the cortical plate even at this date (Juliano et al., 1996; Palmer, 1999).

We observed an unusually high number of terminations in layer 1, in all culture situations except when the cortical piece was obtained at P7. A component that may contribute to this observation is that many of the thalamic pieces were placed adjacent to the pial surface and are therefore nearer to layer 1. In this position, they may more easily find their way to that locus. Although the element of proximity does not completely explain the high number of layer 1 terminations, since they do not occur in the P7 cortical cultures, it may play a role. The proximity of the thalamic piece might be less effective in the P7 cultures since the more mature P7 slices should possess features that result in fewer thalamic terminations in layer 1. This is a possibility, since layer 1 is known to undergo a number of developmental changes that might be more or less encouraging to thalamic afferent termination (Marin-Padilla, 1998).

What is the role of layer 4?

We observed that thalamic terminations were much more focussed in the

normal cortical cultures or those treated with MAM at E38, than in cultures with a poorly formed layer 4 (i.e., treated with MAM at E33). Axons leaving the thalamus preferentially stopped and extended arbors in a region corresponding to layer 4 in normal and E38 MAM-treated organotypic cortical cultures. In the E33 MAM-treated cortical cultures, the thalamic fibers were much more randomly distributed and less likely to extend arbors or branch within any cortical layer. These data suggest that a specific feature of layer 4 provides a stop signal that encourages thalamic axons to halt their growth and arborize as do others (Yamamoto et al., 1989; Bolz et al., 1992; Yamamoto et al., 1992; Molnar and Blakemore, 1995; Yamamoto et al., 1997).

We previously reported in an *in vivo* study that the terminations from the thalamus were almost equally distributed through the remaining cortical layers, in ferrets treated with MAM on E33, rather than focussing in a particular cortical site (Palmer, 1999). The current study supports that finding and also demonstrates that the terminal pattern within layer 4 is highly specific and discrete, a feature not easily discerned using bulk labeling with DiI. The current study used small-localized injections in the thalamic piece that allowed a more detailed analysis of the terminal projections. We were also able to rule out the possibility that the altered terminal projection resulted from MAM treatment affecting the thalamus, rather than an effect of the missing layer 4. This study also observed that in the E33 MAM-treated cultures the afferent thalamic fibers were likely to grow along a cortical layer or through the cortex into the white matter. Such distribution

patterns were not observed in the *in vivo* experiments. This again may be due to the arrangement of the thalamic piece often adjacent to the pia, whereas in the previous study, the thalamic projections were confined by the layer 1 boundary. This suggests that when "forced" to terminate in the in vivo studies, the afferent fibers will do so, in whatever site is available, while in the culture situation, an afferent fiber might choose to grow through the cortex in the absence of layer 4.

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Figure Legends

FIGURE 1. Examples of bisbenzimide staining after different times in culture. Shown in (A) is an acute slice taken at postnatal day 1. Observable is layer 1, a dense undifferentiated cortical plate (CP), and a poorly differentiated layers 5 and 6. (B) is a photomicrograph taken from an organotypic culture of ferret somatosensory cortex that remained 3 days in culture (dic). Clear laminar distinctions can be seen, including the cortical plate (more differentiated than in A), and layers 5 and 6. Shown in C is a photomicrograph, at lower power, of a section of ferret somtosensory cortex that was in culture for 10 days. A laminar pattern is clearly observable in the cortex. Scale bar equals 500 μm.

FIGURE 2. These are examples of dextran injections in organotypic cultures of cerebral cortex. (A) is a photomicrograph of an injection into the intermediate zone. The label shows a typical distribution of a few radial glial cells that extend from the cortical surface to the ventricular zone. (B) is a drawing of a similar injection revealing more planes of focus in the culture. (C) is a photomicrograph of an injection of dextran in a 10 day old culture shown at higher magnification. Emanating from the injection site are apical dendrites, scattered cells can also be seen. Scale bar for A and B equals 500 μm. Scale bar for C equals 100 μm.

FIGURE 3. This is a montage of drawings obtained from cocultures

consisting of thalamic and cortical pieces after different times in culture. Examples are shown for each treatment (normal, E33 MAM treated, and E38 MAM treated) after 3, 5, or 7-10 days in culture (dic). Illustrated in each drawing is the locus of the thalamic piece (shading), the injection site is illustrated with an asterisk. The cortical layers are indicated in each cortical piece. The projections from the thalamic pieces to either normal or E38 MAM treated cortex are substantially more focussed than the terminations into the E33 MAM-treated slices. Scale equals 500 μm.

FIGURE 4. Distributions of the percentages of total labeled terminations in thalamus-cortex cocultures found at different depths in cortical pieces after injections into thalamic pieces. The percent of the total fibers in each group of injections are located to six 100 μm thick bins radiating from the cortical surface. The bin located closest to the cortical surface is numbered 1. Shown here are the distributions for the cocultures composed of P0 normal thalamus and cortical slices obtained from normal, E33, and E38 MAM-treated somatosensory cortex after 3 days in culture. The number of labeled fibers found in bin 2 (100-200 m below the cortical surface) is significantly different from those in the same bin for either the normal ($p=0.038$) or E38 MAM-treated ($p=0.041$) cortex (Mann-Whitney U test). CP, cortical plate.

FIGURE 5. Examples of injection sites in cocultures consisting of P0

normal thalamus and P0, E33 MAM-treated cortex after 5dic. The axons emerging from the thalamus have been highlighted in white. The approximate region of layer 4 is indicated by the arrows. The lower boundary of cortex is indicated by the dashed line. (A) Illustrates numerous fibers growing through cortical plate and into lower regions. (B) Demonstrates the phenomenon observed in cocultures consisting of young cortex, namely the preference of some thalamic fibers for layer 1.

FIGURE 6. Examples of injection sites in cocultures consisting of a slice of thalamus and a slice of cortex. Injection sites and projections from the thalamus can be seen in white. The axons originating from the thalamic injection site are highlighted in white for better visibility. The lower boundary of the cortex is indicated with the dashed lines. The approximate position of layer 4 is indicated with arrows. Shown is an example of a normal, focussed termination (on the left), and an unfocussed pattern of termination from the thalamus in an E33 MAM treated cortical slice (on the right) after 7days in culture.

FIGURE 7. Distributions of the percentages of total labeled terminations in thalamus-cortex cocultures found at different depths in cortical pieces after injections into thalamic pieces. Shown here are the distributions for cocultures of normal thalamus and cortical pieces obtained at P7 for normal and E33 MAM treated animals that remained 3 days in culture. Other conventions and abbreviations as for Figure 4.

FIGURE 8. Distributions of the percentages of total labeled terminations in thalamus-cortex cocultures found at different depths in cortical pieces after injections into thalamic pieces. Shown here are the distributions for cocultures of normal thalamus and cortical pieces obtained at P0 for normal, E38, and E33 MAM treated animals that remained 5 days in culture. Other conventions and abbreviations as for Figure 4. Bins 2 ($p=0.025$) and 3 ($p=0.01$) are significantly different from normal, and bin 3 is also significantly different from the E38 MAM-treated distribution ($p=0.01$) (Mann-Whitney U test).

FIGURE 9. An example of outgrowth from an injection into a thalamic piece. The labeled fibers are growing into an E33 MAM-treated slice for 5 days in culture. The outer border of the cortical piece is indicated with a dashed line. Scale equals 100 μm.

FIGURE 10. Distributions of the percentages of total labeled terminations in thalamus-cortex cocultures found at different depths in cortical pieces after injections into thalamic pieces. Shown here are the distributions for cocultures of normal thalamus and cortical pieces obtained at P7 for normal and E33 MAM treated animals that remained 5 days in culture. Other conventions and abbreviations as for Figure 4.

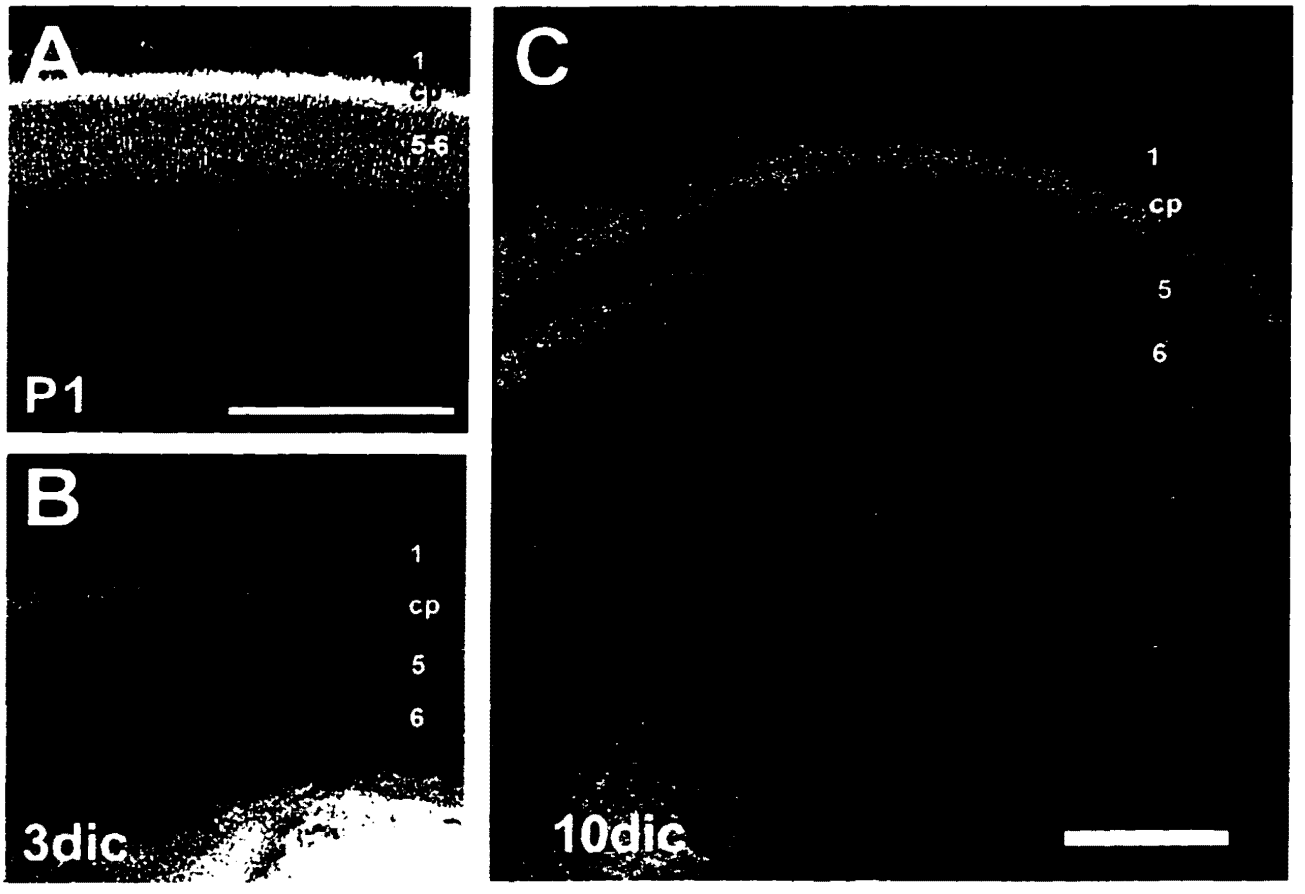
FIGURE 11. Examples of injection sites in cocultures consisting of P0 normal thalamus and P0, normal cortex after 7-10dic. As seen in previous

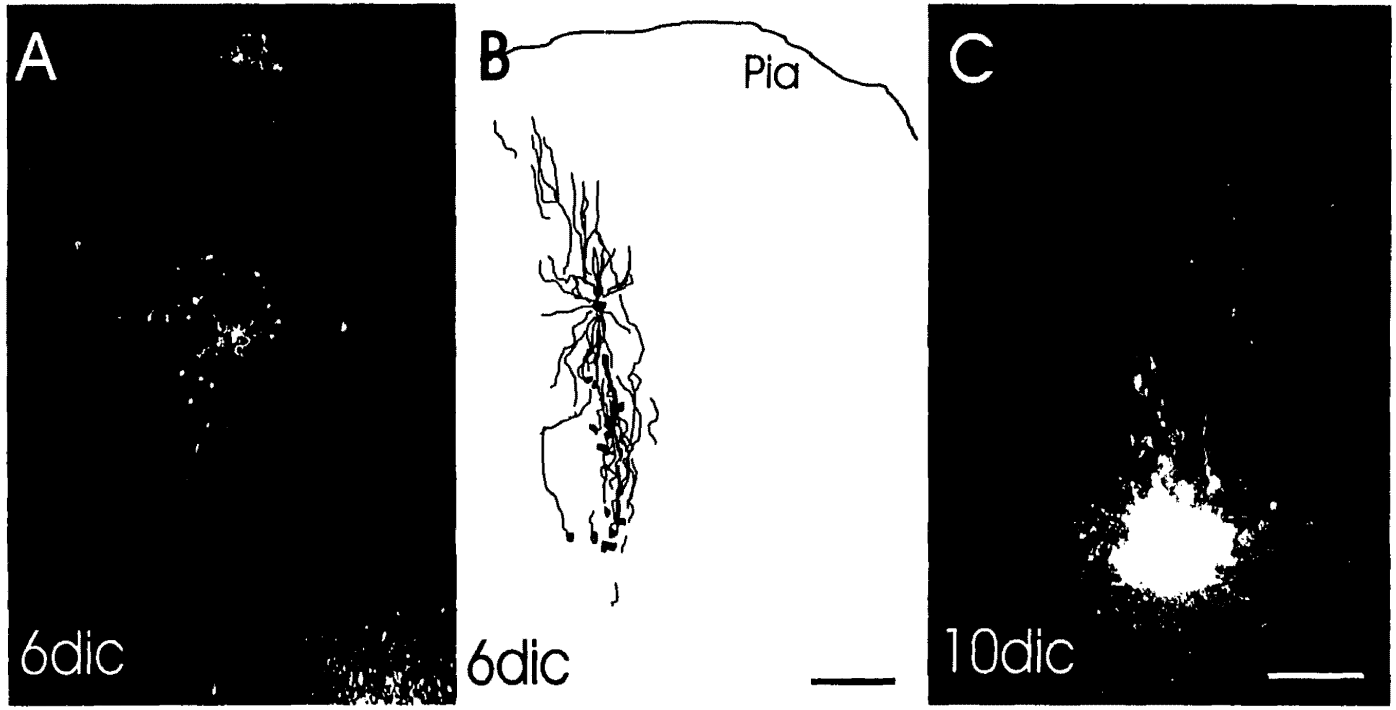
figures, the axons emerging from the thalamus have been highlighted in white. The dashed line indicates the lower boundary of cortex, and the white arrows demarcate the approximate region of layer 4. (A) Illustrates numerous fibers growing into cortical plate. (B) Illustrates that even in normal cortex, some thalamic fibers are found terminating in layer 1.

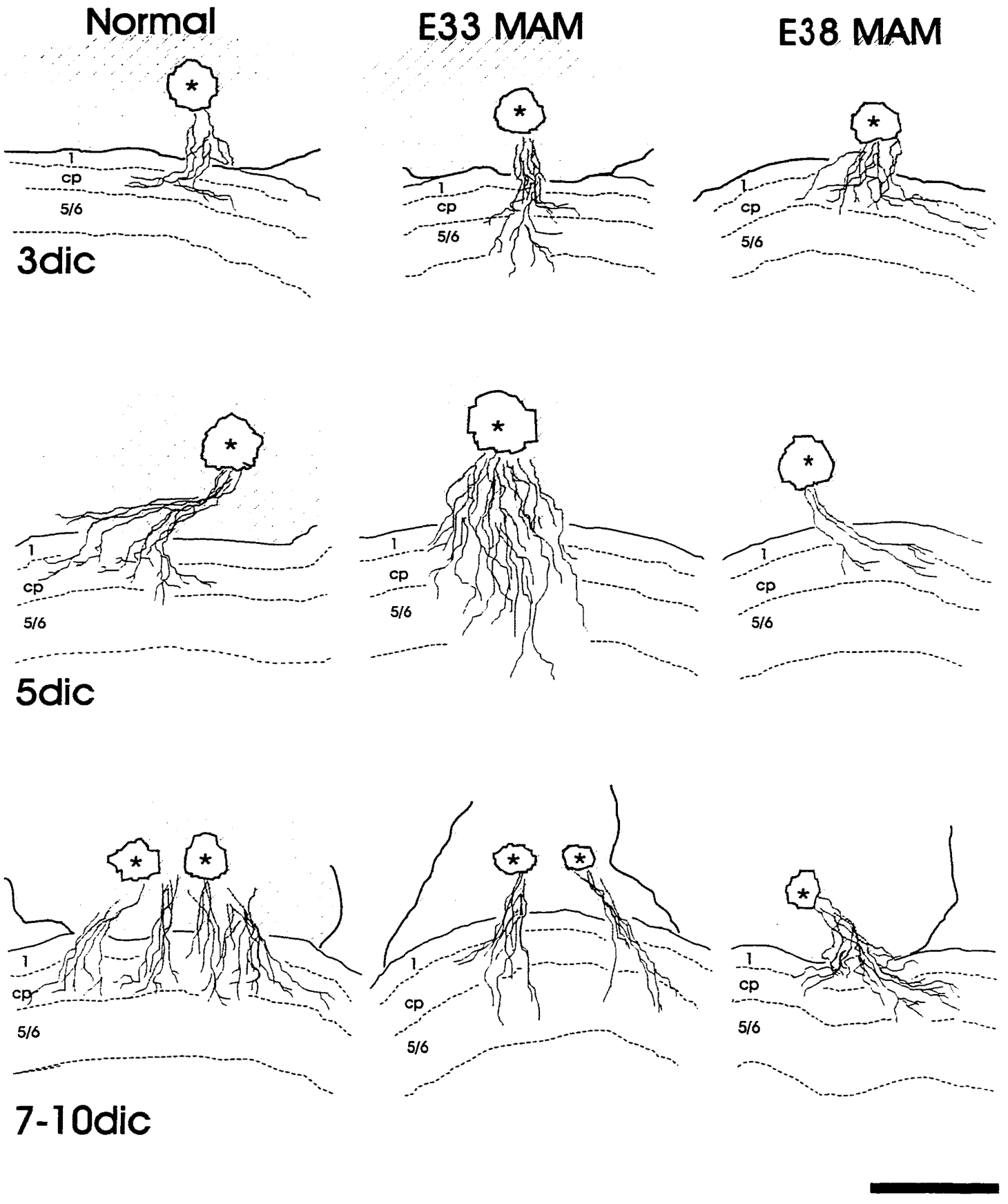
FIGURE 12. Distributions of the percentages of total labeled terminations in thalamus-cortex cocultures found at different depths in cortical pieces after injections into thalamic pieces. The percent of the total fibers in each group of injections are located to six 100 μm thick bins radiating from the cortical surface. The bin located closest to the cortical surface is numbered 1. Shown here are the distributions for the cocultures composed of P0 normal thalamus and cortical slices obtained from normal, E33, and E38 MAM-treated somatosensory cortex after 7-10 days in culture. The number of labeled fibers found in bin 2 ($p=0.014$) and bin 4 ($p=0.047$) are significantly different from those in the same bin for the normal distribution or for the E38 MAM-treated distributions bin 2 ($p=0.03$) and bin 4 ($p=0.039$) (Mann-Whitney U test). CP, cortical plate.

FIGURE 13. Distributions of the percentages of total labeled terminations in thalamus-cortex cocultures found at different depths in cortical pieces after injections into thalamic pieces. Shown here are the distributions for cocultures of normal thalamus and cortical pieces obtained at P7 for

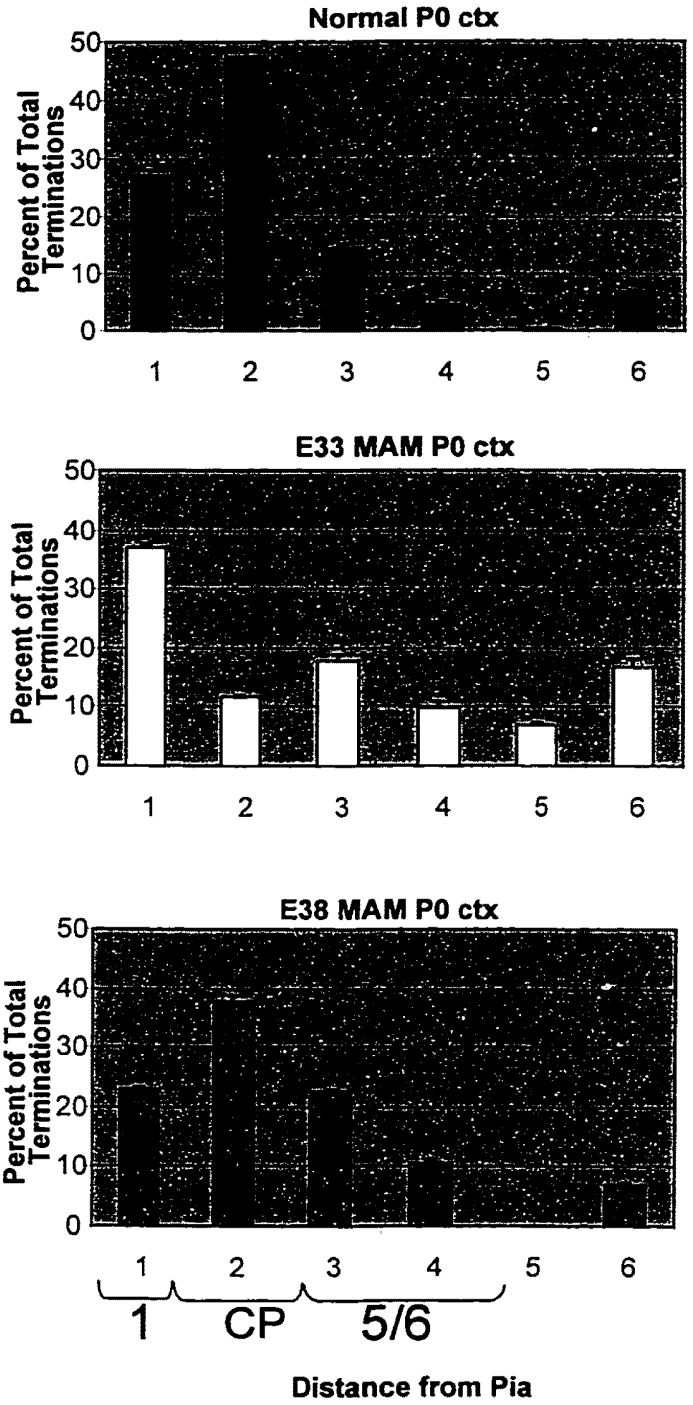
normal and E33 MAM treated animals that remained 7-10 days in culture. Other conventions and abbreviations as for Figure 4.

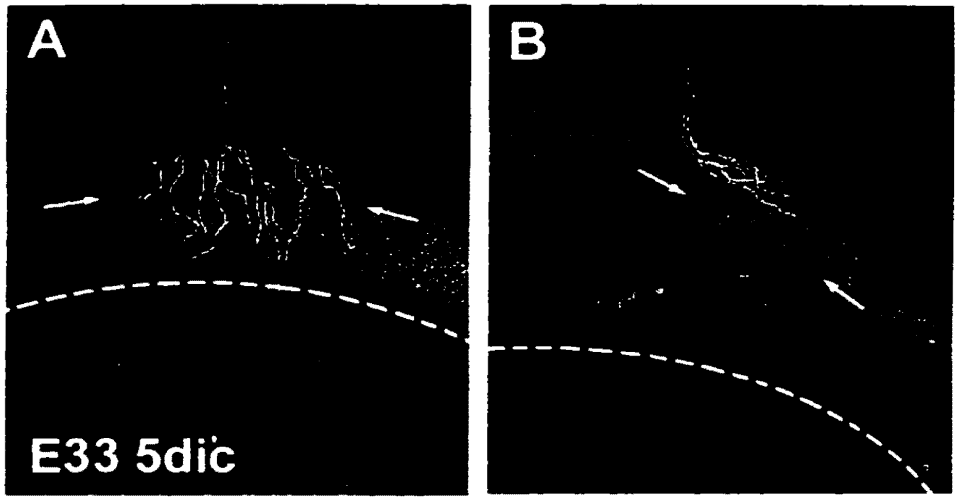

A
1
cp
5-6
P1
B
1
cp
5
6
3dic
C
1
cp
5
6
10dic

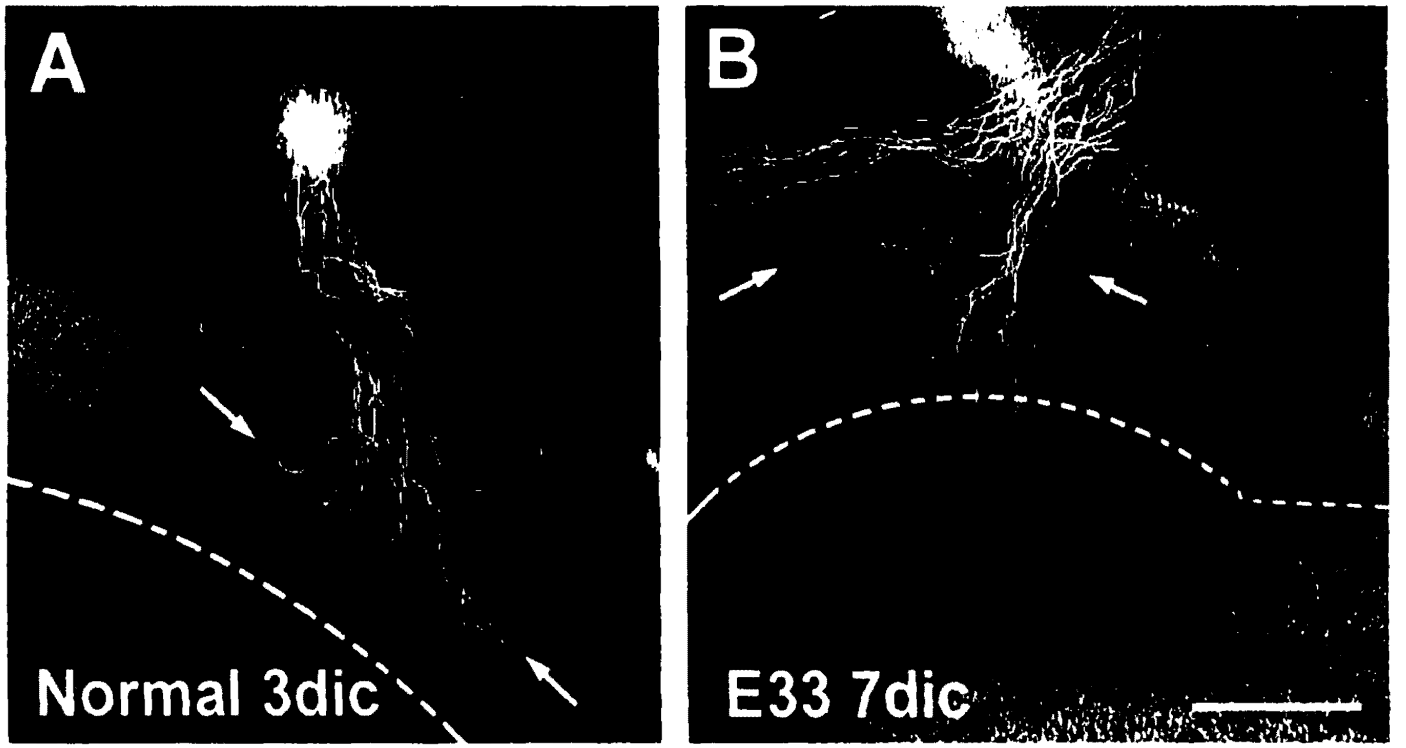

A
6dic
B
Pia
6dic
C
10dic


Normal
E33 MAM
E38 MAM
3dic
5dic
7-10dic
1
cp
5/6

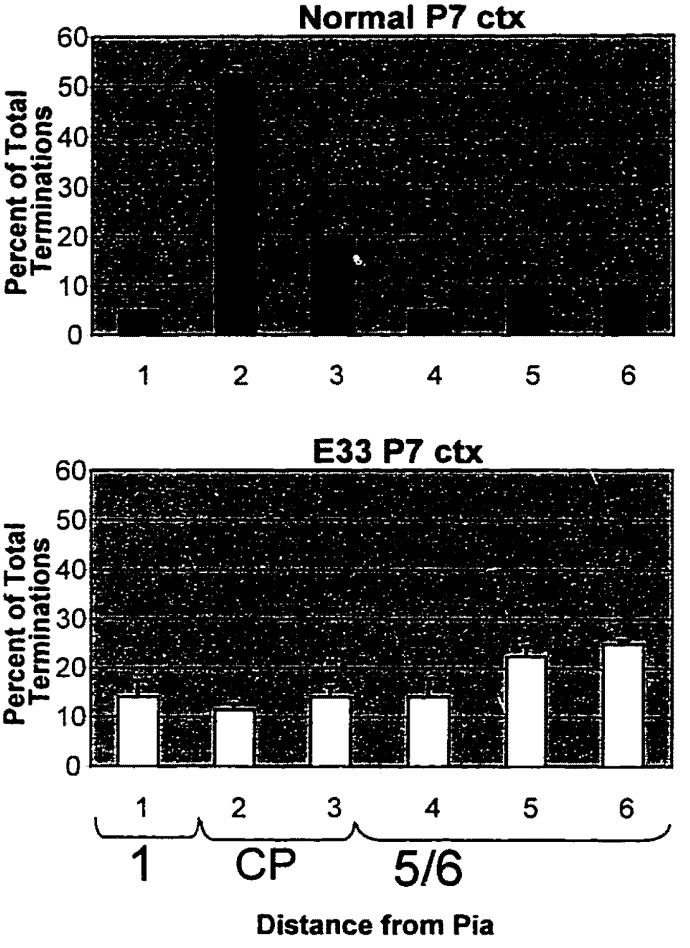
3 DIC


Normal P0 ctx
Percent of Total Terminations
50
40
30
20
10
0
1
2
3
4
5
6
E33 MAM P0 ctx
Percent of Total Terminations
50
40
30
20
10
0
1
2
3
4
5
6
E38 MAM P0 ctx
Percent of Total Terminations
50
40
30
20
10
0
1
2
3
4
5
6
1
CP
5/6
Distance from Pia

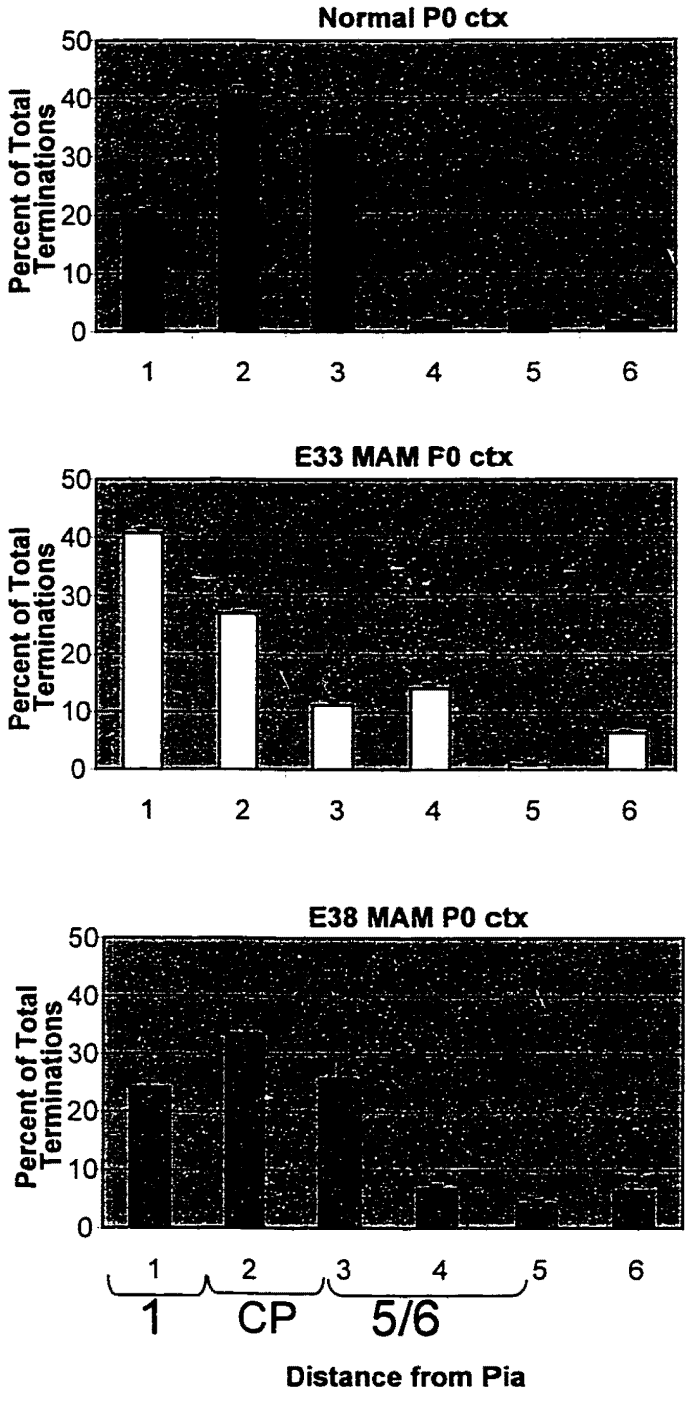

A
B
E33 5dic


A
Normal 3dic
B
E33 7dic

3 DIC


Normal P7 ctx
Percent of Total Terminations
60
50
40
30
20
10
0
1
2
3
4
5
6
E33 P7 ctx
Percent of Total Terminations
60
50
40
30
20
10
0
1
2
3
4
5
6
1
CP
5/6
Distance from Pia

5 DIC


Normal P0 ctx
Percent of Total Terminations
50
40
30
20
10
0
1
2
3
4
5
6
E33 MAM P0 ctx
Percent of Total Terminations
50
40
30
20
10
0
1
2
3
4
5
6
E38 MAM P0 ctx
Percent of Total Terminations
50
40
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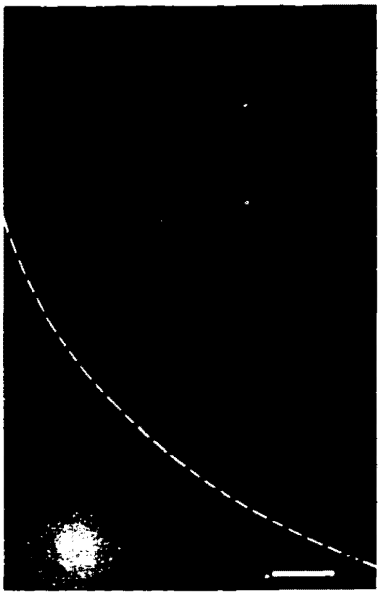
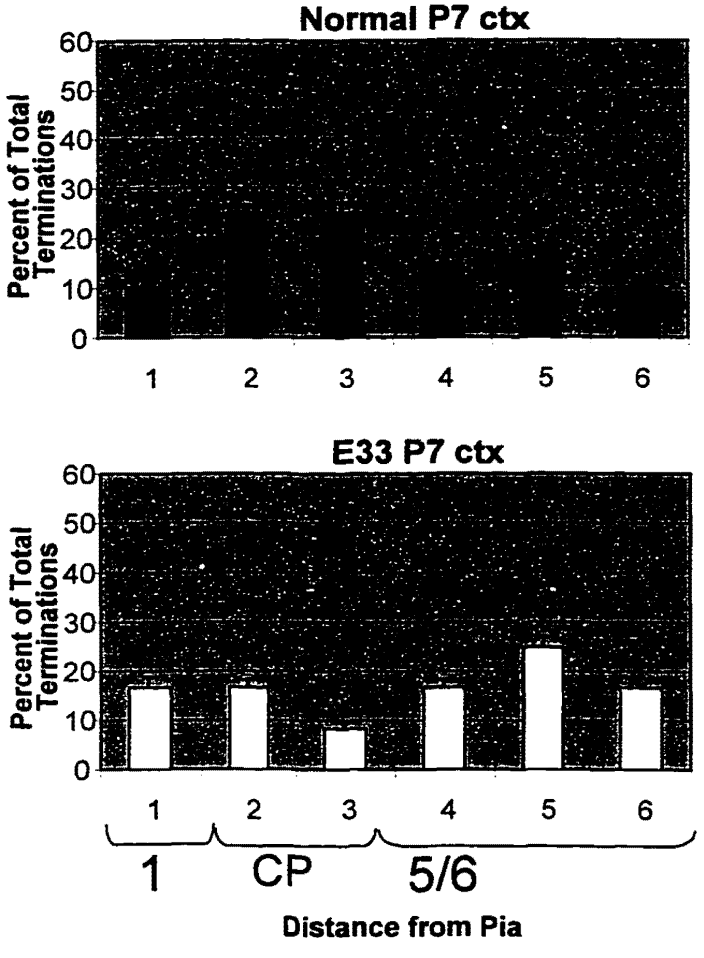
3
4
5
6
1
CP
5/6
Distance from Pia



5 DIC


Normal P7 ctx
Percent of Total Terminations
60
50
40
30
20
10
0
1
2
3
4
5
6
E33 P7 ctx
Percent of Total Terminations
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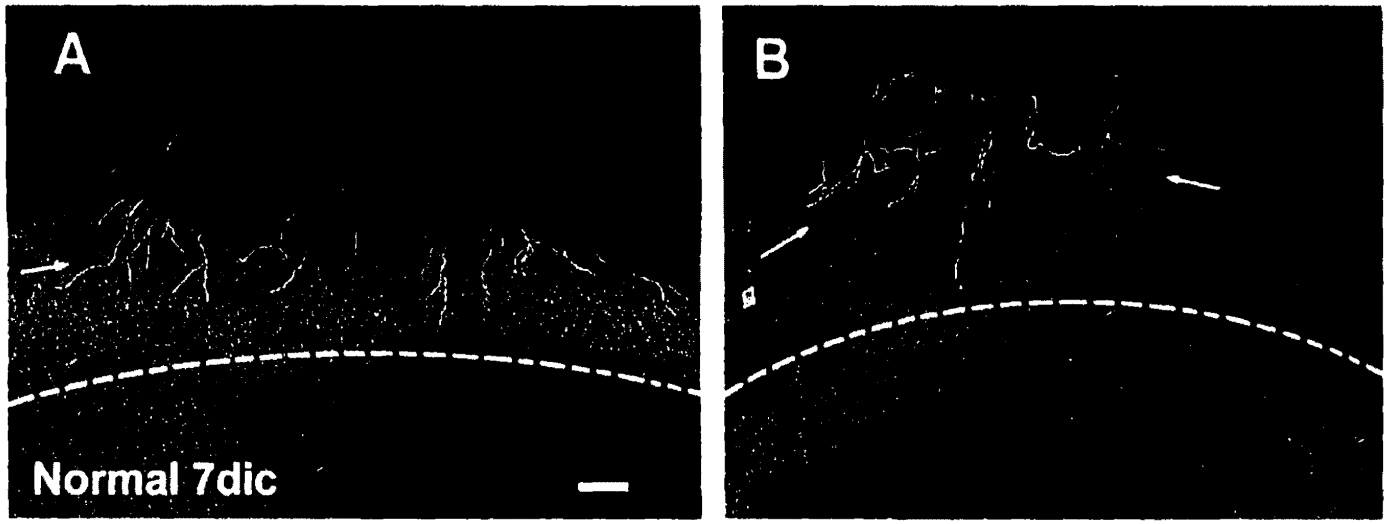
3
4
5
6
1
CP
5/6
Distance from Pia


A
Normal 7dic
B

7-10 DIC

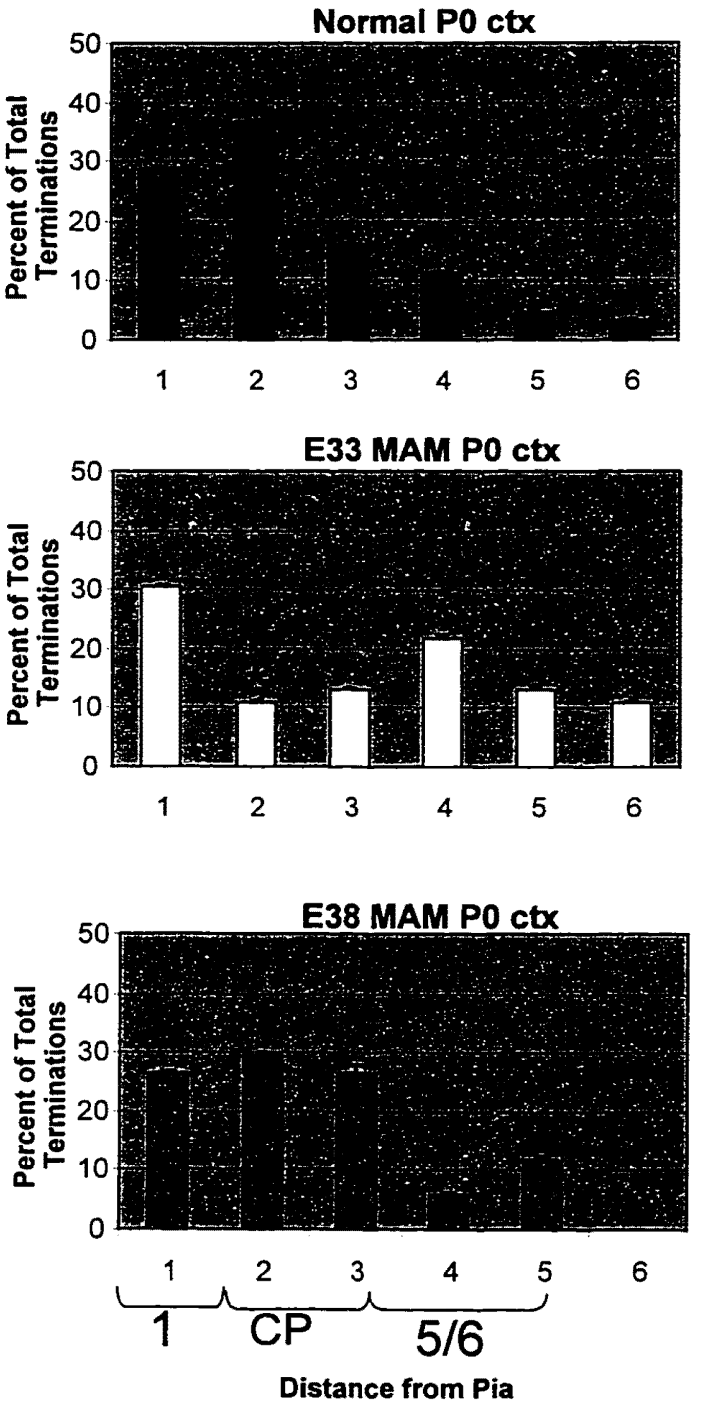

Normal P0 ctx
Percent of Total Terminations
50
40
30
20
10
0
1
2
3
4
5
6
E33 MAM P0 ctx
Percent of Total Terminations
50
40
30
20
10
0
1
2
3
4
5
6
E38 MAM P0 ctx
Percent of Total Terminations
50
40
30
20
10
0
1
2
3
4
5
6
1
CP
5/6
Distance from Pia

TABLE 1

Number of Pregnant Ferrets

Normal	E33 MAM-Treated	E38 MAM-Treated
12	6	3

Number of Kits

Normal	E33 MAM-Treated	E38 MAM Treated
32	15	7

Cortical Cultures Analyzed

	3dic	5dic	7-10dic
Normal	8	6	6
E33	3	2	

Cortical Cultures Paired with Normal P0 Thalamus

Normal Cortex - P0	35
E33 MAM cortex - P0	50
E38 MAM Cortex - P0	37
Normal Cortex - P7	9
E33 MAM Cortex - P7	12

Cocultures Analyzed - All Paired with P0 Normal Thalamus

	3dic	5dic	7-10dic
Normal	8	7	7
E33	12	9	7
E38	6	5	4

Summary

Thalamocortical development is a complex process involving the interrelated growth and development of two spatially separate portions of the brain. Not only must thalamic neurons grow into the cortex, but they must also do so with amazing precision. Clusters of thalamic nuclei map to distinct regions of the cortex, thalamic terminations identify, arborize, and synapse on appropriate target cell layers. How these developing thalamic fibers grow into the cortex and terminate within the appropriate target layers with such specificity are beginning to be clarified.

Factors Involved in Thalamic Growth and Target Selection

The growth and differentiation of thalamic afferents into the neocortex may involve at least three components: (1) The presence of guidance or attractant molecules on the path to or within the neocortex; (2) the composition of the substrate upon which the axons grow and (3) a stop signal within the target layer that instructs thalamic axons to stop growing longitudinally and begin arborizing.

1) Presence of Guidance Molecules. These molecules influence neurites, not only by attracting axons into appropriate areas but also by repelling them from inappropriate sites. The Pax-6 gene has been implicated in the development and guidance of thalamic afferents along the thalamocortical pathway, particularly early on in thalamocortical development (Kawano et al., 1999). In mice without the gene coding for Pax-6 thalamic afferent axons failed

to extend into appropriate cortical regions from the outset. Recent experiments by Miller et al. (1995) suggest a possible role for chondroitin sulfate proteoglycans (CSPG) as another molecular signal further along the thalamocortical pathway. CSPG forms inhibitory boundaries to axons in the peripheral nervous system (Snow et al., 1990; McKeon et al., 1991; Bovolenta et al., 1993). The molecules also promote neurite growth in tissue culture (Iijima et al., 1991; Lafont et al., 1992; Bicknese et al., 1994). One core protein of the CSPG family, neurocans, was shown by Miller and Pearlman to be richly expressed by the subplate during the time of thalamic axon accumulation. Later, within the cortex, neurocans expression was observed to increase in developing cortical plate coinciding with the growth of thalamic axons (Miller et al., 1992; Fukuda et al., 1997). These results suggest that there may be a cascade of molecules expressed at different spatial and temporal intervals along the thalamocortical pathway to guide the thalamic afferents into their appropriate cortical region. Our research demonstrates that following MAM treatment thalamic afferents arrive in appropriate cortical areas, therefore, the expression of these molecules does not appear to be disrupted by our MAM model.

2) Composition of Substrate. A second component of proper innervation may involve the substrate upon which the axons move. Adhesive molecules such as laminin, appear to be important and necessary for thalamic afferent growth and are richly and transiently expressed in thalamic pathways (Hubener et al., 1995). While these molecules may not "guide" the growing afferent per se,

cell adhesion to a substrate is critical to their ability to invade the cortex (Doherty and Walsh, 1994). Several other molecules have been shown to increase during thalamocortical invasion, including the adhesion molecules cytotactin and L1. Cytotactin is most prevalent initially in the subplate but later becomes apparent in the cortical plate as it undergoes differentiation (Sheppard et al., 1991). L1 promotes neuron-neuron adhesion and has been demonstrated by Fukuda et al.(1997) to be expressed along the thalamocortical pathway, including lower cortical plate. Additional adhesive factors identified in the deeper cortical layers have also been shown to control thalamic axon growth (Emerling and Lander, 1994).

3) Presence of a Stop Signal. The third component of thalamocortical innervation may involve a "stop" signal. Molnar and Balkemore (1995) suggest that thalamocortical afferents might recognize a molecular stop signal associated with layer 4, which by itself or in association with other signals bring about layer-specific axonal arborization. This signal causes the arriving afferent to change its phenotype from the "extending" neuron to the "connecting" neuron, stop linear growth and begin the precise branching necessary for formation of synapses with layer 4 cells. In coculture experiments pairing cortex with thalamus, Bolz et al. (1990) demonstrated that thalamic afferents invaded the cortex from either the pial or white matter surfaces and formed appropriate terminations in the middle of the cortical plate. These findings are supported by a number of other coculture experiments that likewise demonstrate that thalamic

afferents terminate in a laminar specific fashion within layer 4 (Yamamoto et al., 1989; Yamamoto et al., 1992; Yamamoto et al., 1997; Molnar and Blakemore, 1999). These results along with our own seem to definitively localize the stop signal to the vicinity of layer 4. There are many possibilities as to the nature of this stop signal. Potential "stop signal" candidates include: the presence of inhibitory molecules, neurotrophic factors, or a cell surface molecule on the target cell.

The suggestion that the stop signal may be an inhibitory factor specifically limiting thalamic extension into regions where the factor is being expressed, while not preventing branching and extension in layers where the factor is absent. This is supported by the finding that differential expression of semaphorin family molecules occurring in the maturing supragranular layers might limit the growth of thalamic fibers into layers superior to layer 4 (Skaliora et al., 1998). While these molecules may be expressed in supragranular layers, other researchers have shown that the semaphorin family possess both chemoattractive as well as chemorepellant properties (Bagnard et al., 1998). Furthermore, nany researchers have demonstrated that thalamic afferents stop within their correct target when thalamic explants have been juxtapostioned against the pial surface in the coculture environment just as they do *in vivo* (Yamamoto et al., 1989; Bolz et al., 1990; Yamamoto et al., 1992; Yamamoto et al., 1997; Molnar and Blakemore, 1999).

Several investigators report that members of the neurotrophin family

promote axonal and dendritic arborization in the CNS, including the neocortex (Cabelli et al., 1995; McAllister et al., 1995). Exogenous administration of BDNF or NT-4/5 does not alter the laminar localization of axonal innervation suggesting that these factors may control growth and elaboration of branches rather than restricting branches to a precise laminar targets (Cabelli et al., 1995).

Of the candidate "stop" mechanisms proposed, the most likely remains a cell surface molecule. Gotz et al.(1992) proposed the contact of the thalamic afferent may initiate a mechanism to alter the cytoskeleton of the thalamic afferent and begin arborization and formation of axonal endings. Yamamoto et al (1997), suggest that stopping and branching behavior of thalamic axons in layer 4 is a result of target-finding processes in which thalamic afferents stop and branch after crossing the boundary into layer 4 and making initial contact with layer 4 cells.

None of these finding preclude the possibility that the stop signal may not be a simple one-molecule mechanism, but a sequence of molecules, markers or signals all of which combined cause the phenomenon of thalamic afferents to specifically and preferentially terminate in layer 4.

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